THE STUDY OF THE DENATURATION OF SQUASH SEED GLOBULIN

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A STUDY OF THE DENATURATION OF SQUASH SEED GLOBULIN

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INTRODUCTION

Relatively little attention has been given the protein squash seed globulin in Biochemical and Physiological experimentation although it was one of the first globulins to be isolated and identified as a protein of this type. The study of the globulin was resumed a few years ago when it received prominence as a substitute for edestin, which was becoming less available as the restrictions on the growing of hemp seed increased. A waste product of the canning industry in some states, squash seed globulin is always on the market at a price that is usually a small fraction of that commanded by most cucurbit seeds. The methods of isolation of the globulin are quite uninvolved and the yield fairly high.

A study of the denaturation of squash seed globulin was undertaken in order to record more data on the production of sulfhydryl groups by various reagents, and to determine the effect of sugars on the treatment of the globulin with these reagents. As more information is accumulated on the properties and reactions of squash seed globulin, a better understanding of the role it plays in animal physiology will be realized, and the chemistry of the proteins of both plants and animals will be brought into closer relationship.

In the historical section of this thesis an attempt

was made to submit all the literature pertaining to the protein squash seed globulin. In view of the nature of the problem emphasis was placed on those parts of the literature dealing with denaturation and the content of sulfur and sulfur-containing amino acids in squash seed globulin.

HISTORICAL

In the review of the literature pertaining to this problem, consideration will be given to the following topics:

1. The isolation, analyses, and properties of squash seed globulin.
2. Methods of estimating sulfhydryl groups in proteins.
3. The influence of carbohydrates on the coagulation of proteins.

ISOLATION OF SQUASH SEED GLOBULIN

The main protein isolated from the seeds of squash, Cucurbita maxima, is known as squash seed globulin or cucurbitin. It is soluble in saline solutions but insoluble in water, can be precipitated from saline extracts of the seeds by dilution or by dialysis, and therefore is termed a globulin protein according to Osborne's classification (1).

Squash seed globulin was first prepared and analyzed in 1878 by Barbieri (2) whose preparations were not, however, crystalline. In 1881 Grübler (3) first applied the method of allowing warm sodium chloride solutions saturated with squash seed globulin to cool in order to obtain octahedral crystals. Chittenden and Hartwell (4)

later prepared the protein in the crystalline form and published an analysis of it.

With the exception of Osborne's early work (5) on seed globulins no attempt was made to improve the methods of preparing squash seed globulin until 1939. Krishnan and Krishnaswamy (6) used 10% sodium chloride solution to remove the globulin, then diluted this extract with 15 volumes of water at 40° in order to bring about precipitation and crystallization.

The difficulty of obtaining hemp seed during the last few years instigated a change over to squash seed as a readily available source of seed globulin in place of edestin. This substitution was reported by Vickery, Smith, and Nolan (7) and later worked out in detail by Vickery, Smith, Hubbell, and Nolan (8). Their method of preparation of squash seed globulin included the use of 10% sodium chloride for extraction purposes, but specified dilution of the extract with four volumes of water at 60° to obtain crystalline globulin. Preheating of the squash seed extract to 75° and subsequent filtration before dilution ensured the removal of any albumins present. Slow cooling of the diluted solution from 60° made for larger crystals, which could either be collected and dried, or kept under 2% sodium chloride solution for some time.

ANALYSES OF SQUASH SEED GLOBULIN

Barbieri (2) made the first elementary analysis of squash seed globulin on completion of its preparation. His value for sulfur content of the amorphous protein was 0.60%. Later Grübler (3), Chittenden and Hartwell (4), and Osborne (5) completed analyses of the crystalline protein with generally agreeing results. Their values for percent sulfur were:

Grübler	0.96
Chittenden and Hartwell	1.01
Osborne	0.89
Average sulfur content	0.95

Not until 1923 was further significant analysis done on squash seed globulin. Jones and Gersdorff (9) isolated the crystalline globulin from cantaloupe seed, Cucumis melo, and made a comparative study of this protein with the crystalline globulin of the squash seed. Their comparison of the nitrogen distribution indicated a great similarity between the two globulins. The analysis of squash seed globulin yielded a sulfur content of 1.00% on a moisture and ash-free basis. Analysis of the basic amino acids of squash seed globulin yielded a value of 1.26% for cystine. No difference in the crystallographic or optical properties of the two globulins was detected.

Similar comparative work was done on the seed globulins from pumpkin, squash, watermelon, and cucumber by Smith, Greene, and Bartner (10). Their tables of amino acid composition indicated small differences in the varieties of seed globulins, as well as differences between edestin and the crystalline globulin of the tobacco seed.

Hess and Sullivan (11) investigated the cysteine, cystine, and methionine content of squash seed globulin. Determinations of the sulfhydryl groups present in the unhydrolyzed protein were compared with the cysteine determined in the acid hydrolysate of the same protein and the resulting values were found to agree. According to Hess and Sullivan, this finding indicated that cysteine complexes were present in the native protein. The cysteine content of the unhydrolyzed protein in the native state was determined by direct titration with iodine according to the Okuda method for cysteine. The same value was obtained whether the titration was carried out in the presence of 8 millimoles of guanidine hydrochloride per ml. of solution or in its absence, since the sulfhydryl groups of proteins can be titrated by iodine whether the protein is in the native or denatured state.

In squash seed globulin cysteine, cystine, and methionine accounted for 87% of the total sulfur of the

unhydrolyzed proteins. The sulfur-containing amino acids, however, accounted for all of the sulfur in the hydrolysates of squash seed globulin. About 13% of the sulfur is lost during the hydrolysis of this protein in a form not yet explained.

Using the following hydrolyzing agents, Hess and Sullivan's results for squash seed globulin were:

Hydrolyzing Agent	%Cysteine	%Cystine
20% HCl and Urea	0.49	0.82
20% HCl	0.17	1.14
6N H_2SO_4	0.35	0.97

By direct titration with iodine: cysteine 0.51%

Total sulfur in unhydrolyzed protein: 0.99%

Cysteine, cystine, and methionine sulfur: 0.86%

Further work on squash seed globulin was published by Hess and Sullivan (12), in which the phenylalanine content of the protein was determined colorimetrically. Hydrolyzing agents employed were: 7N H_2SO_4, 20% HCl, and 5N NaOH.

Smith and Greene (13) completed a more recent study of the amino acid composition of squash seed globulin. The following sulfur distribution in squash seed globulin was reported:

Cystine	1.11%
Cystine sulfur	0.30%
Methionine sulfur	0.50%
Total sulfur (calculated)	0.80%
Total sulfur (analysis)	0.95%

PROPERTIES OF SQUASH SEED GLOBULIN

In his initial investigations of squash seed globulin in 1892, Osborne (5) found that in distilled water, either at 20° or 60° the protein, whether separated from solution in sodium chloride brine by dialysis or by cooling, was wholly insoluble. It was also insoluble in strong or diluted glycerin, cool or warmed to 60°. In 10% salt solution the crystals dissolved with the exception of a mere trace, and the resulting solution was not precipitated by saturating with sodium chloride, but was completely precipitated by saturating with ammonium sulfate or magnesium sulfate. In 0.1% sodium carbonate and 0.01% potash solutions the protein dissolved readily and completely; these solutions were not precipitated by sodium chloride. When dissolved in 10% sodium chloride solution and heated to coagulum, the protein behaves as follows: turbidity at 87°; flocculation at 95°; and after filtering and boiling, no coagulation. However, on adding a drop of

acetic acid, the filtered solution yielded a large precipitate.

Osborne and Harris (14) reported their work on the specific rotation of squash seed globulin - $[\alpha]_D^{20} = 38.73^\circ$. Jones and Gersdorff (9) determined the refractive index of the globulin - N_D^{20} = approx. 1.545. An investigation of the isoelectric points of acetylated and deaminated cucurbitin was carried out by Kizel and Jurkevitsch (15). The isoelectric point of cucurbitin was reported pH 6.3.

The majority of the information on squash seed globulin has been supplied by Osborne (1) in his monograph on seed globulins. In this publication the properties of seed globulins in general were recorded on the assumption that most proteins of this origin were similar in physical and chemical behaviour. The properties that can safely be attributed to squash seed globulin from Osborne's work are listed below. Most of the globulins from seeds, unlike those of animal origin, are incompletely coagulated by heating their faintly acid solutions, and in some cases are not coagulated at all. The degree of solubility of many seed globulins in sodium chloride solution depends much on the temperature of the solution and increases rapidly at 30°. Their solubility in salt solutions is much affected by the amount of acid present; in general, the greater the quantity of acid, the stronger the saline

solution necessary to dissolve the globulin. Precipitation of seed globulins by acids may be due either to the acid uniting with a base previously combined with the protein to form a soluble compound, or by the formation of a salt of the protein insoluble in water. Many of the seed proteins appear to be little affected by long treatment with alcohol, with little or no evidence of change caused. Certain globulins pass into insoluble modifications, the proteans, on standing in contact with pure water, or in the presence of hydrogen ions.

METHODS OF ESTIMATING SULFHYDRYL GROUPS IN PROTEINS

While the term "denaturation" has become firmly implanted in the literature, the definition of this process has been very loose and subject to many changes and revisions. In the present thesis the following definition as proposed by Neurath, Greenstein, Putnam, and Erickson (16) can appropriately be assigned to "denaturation": Denaturation is any non-proteolytic modification of the unique structure of a native protein, giving rise to definite changes in chemical, physical, or biological properties. This definition excludes processes which result in the hydrolysis of peptide bonds, i.e., chemical or enzymatic degradation.

The literature on the liberation of sulfhydryl groups during the denaturation of proteins and the occurrence of sulfhydryl groups in proteins has been amply covered by Duddles (17) and by Hardt (18). Hardt has also reviewed the work on the methods of estimating sulfhydryl groups in proteins up to the work of Anson and Mirsky (19) (20) (21), and of Anson (22) (23).

Continuing his work with the sulfhydryl groups of proteins, Anson (24) investigated both the nitroprusside and the ferricyanide reactions on egg albumin denatured with urea and with guanidine hydrochloride. He observed that the amount of ferricyanide reduced to ferrocyanide by the sulfhydryl groups of denatured egg albumin was, within widely separated limits, independent of the ferricyanide concentration. Sulfhydryl groups could be abolished by reaction of the native form with iodine. Furthermore, it was possible to oxidize all the sulfhydryl groups with iodine without oxidizing many of them beyond the disulfide stage.

Mirsky (25) experimented with different denaturing agents on egg albumin, using the nitroprusside and iodoacetamide reactions, as well as the ferricyanide reaction to estimate the number of sulfhydryl groups formed upon denaturation. He noted that in neutral medium, ferricyanide appeared to react only with the sulfhydryl groups of egg

albumin; therefore the quantity of ferrocyanide formed could be considered the equivalent of the number of sulfhydryl groups in egg albumin detectable with nitroprusside. In solutions of urea, guanidine hydrochloride, and Duponol PC (in excess) the same number of sulfhydryl groups were formed.

THE INFLUENCE OF CARBOHYDRATES ON THE COAGULATION OF PROTEINS

The interaction between carbohydrates and proteins has been suspected as the underlying common basis for a wide variety of naturally occurring phenomena, many of which play important roles both in biological and in industrial processes. Investigators have attempted to gain more knowledge of this interaction by studying the denaturation of proteins as influenced by carbohydrates.

In 1929, Beilinnson (26) reported the inhibition of the heat coagulation of proteins by sucrose. Egg albumin was found to be thermostable at 75° in a solution saturated with sucrose. Glycerol also provided protection from heat coagulation although the effect was not as great as from sucrose. Newton and Brown (27) later showed that sucrose and glucose prevented the denaturation or coagulation of plant sap proteins by freezing.

Brosteaux and Eriksson-Quensel (28) found that when the

proteins hemoglobin, serum albumin, serum globulin, edestin, phycoerythrin, erythrocruorins, and hemocyanins were dried in vacuum at room temperature, they were more or less denatured, and did not assume their former state of dispersion when placed in water. The addition of sugars, sodium chloride, alanine, glycine, or gelatin to the proteins before drying protected them to some extent from denaturation, the effectiveness of the substances decreasing in the order given. Urea was practically ineffective, while lactose was the most effective. The minimal quantities found by experiment to prevent denaturation completely closely approached the quantities calculated necessary to form a unimolecular layer on the surface of the protein micelles.

Ball, Hardt, and Duddles (29) found that D-glucose, D-fructose, D-mannose, L-arabinose, D-xylose, D-mannitol, and sucrose had a preventative effect on the heat coagulation of egg albumin. The inhibiting effect did not increase with the increase of time of contact of the agent with the egg albumin even at a high pH. The influence of sugars and mannitol on the liberation of sulfhydryl groups from egg albumin was determined by the use of both the iodoacetic acid method and the phenolindo-2,6-dichlorophenol titration method for the estimation of liberated sulfhydryl groups. The influence of sugars on the heat

coagulation of proteins was studied by determining the amount of nitrogen left in the filtrate from the heat coagulated protein solution.

Using electrophoretic procedures, Hardt, Huddleson, and Ball (30) found that D-glucose, D-galactose, L-arabinose, sucrose, D-fructose, lactose, D-mannitol, and D-xylose all showed some degree of inhibiting action against the C component formation in heated bovine serum. The C component was formed by heating an albumin-alpha-globulin fraction of bovine serum.

Fischer (31) discovered that D-ribose, L-arabinose, ascorbic acid, and digitoxose prevented the heat coagulation of bovine serum at a concentration of 5% by volume. Glucose, sorbose, galactose, fructose, glucosamine, inositol, maltose, lactose, sucrose, raffinose, glycogen, and soluble starch had no effect on bovine serum at the same concentration. The preventative action of ascorbic acid appears to be the result of its acidity since its sodium salt has no inhibiting effect.

EXPERIMENTAL

The experimental work on this problem was separated into two parts:

1. An attempt to liberate sulfhydryl groups from squash seed globulin
   - A. Method
   - B. Reagents
   - C. Preliminary experiments
   - D. Experiments on squash seed globulin
   - E. Nitroprusside tests
   - F. Iodine titration of squash seed globulin.
2. The effect of sugars on the coagulation of squash seed globulin
   - A. Coagulation by hydrochloric acid
   - B. Coagulation by heat.

AN ATTEMPT TO LIBERATE SULFHYDRYL GROUPS FROM SQUASH SEED GLOBULIN

A. METHOD:

Protein sulfhydryl groups are estimated by means of their reaction with ferricyanide, as a result of which they are oxidized to disulfide groups and ferrocyanide is formed (25):

2 protein SH + 2 ferricyanide = protein S-S + 2 ferrocyanide.

An excess of ferricyanide is added and the quantity of

ferrocyanide is estimated. This is done by adding ferric sulfate which reacts with ferrocyanide to form Prussian blue which is estimated in a photoelectric colorimeter. The intensity of the blue color is a measure of the number of active, protein sulfhydryl groups.

For relatively simple sulfhydryl compounds such as cysteine and glutathione, the reaction with ferricyanide proceeds stoichiometrically (32). Conditions under which the sulfhydryl groups have reacted with ferricyanide in a precise and definite manner have been found (25). The protein should be dissolved in approximately neutral solutions of the denaturing agent. Under these conditions the reaction proceeds rapidly. It is completed in less than one minute; no more ferricyanide is reduced in 60 minutes than in one minute. Nor within wide limits do the concentration of ferricyanide or the temperature affect the quantity of ferricyanide reduced.

B. REAGENTS:

The experimental work was based on Anson's procedure (24), and an attempt made to outline a method for the estimation of sulfhydryl groups produced on the denaturation of squash seed globulin using the reagents listed below.

Protein: Finely ground squash seeds, Cucurbita maxima, were used as a source of squash seed globulin. Three

different preparations of the globulin, all isolated according to the method of Vickery, Smith, Hubbell, and Nolan (8), were employed in this problem. All preparations were crystallized three times. The first preparation, isolated in 1945, was kept in a dry condition until used subsequently in 10% sodium chloride solution. Difficulty was encountered in putting this dried globulin into 10% sodium chloride solution. About one quarter of the amount used would not dissolve and appeared to be denatured, probably due to the methods of washing and drying the protein during isolation. The second and third preparations were isolated currently and left in the refrigerator as crystals under 2% sodium chloride solution and under toluene. This globulin went back into saline solution with little or no evidence of precipitated, denatured globulin. Before making nitrogen determinations all globulin solutions were filtered slowly through paper pulp. The following squash seed globulin solutions, all 10% in respect to sodium chloride, were used in this problem:

Protein I: made up from the dried globulin prepared in 1945. Protein content: 11.61 mg./ml.

Protein II: made up from the second protein preparation kept as crystals under 2% sodium chloride. Protein content: 3.79 mg./ml.

Protein III: made up from the third protein preparation kept under 2% sodium chloride. Protein

content: 9.79 mg/ml.

Protein II was used for most of the qualitative side experiments in the problem. The nitrogen content of each protein solution was determined in triplicate by Microkjeldahl procedure (33). Two ml. aliquots of each solution were used and the boric acid distillates titrated with 0.0205M hydrochloric acid, using one drop of methylene blue and eight drops of methyl red as an indicator. From the nitrogen content, the protein concentration of each solution was determined , using the value 17.05% for the nitrogen content of squash seed globulin (9).

Sodium Chloride: The protein was kept in 10% (or 1.67M) sodium chloride solution to prevent its precipitation in the crystalline form. However, at 20° it will stay in solution under 5% sodium chloride. The reacting solutions were maintained at 10% sodium chloride concentration until after ferricyanide was added; then the salt content is irrelevant since denaturation is complete.

Cyteine Hydrochloride: Cysteine was chosen as a suitable sulfhydryl reagent to test the validity of the method proposed for the liberation of sulfhydryl groups from squash seed globulin. A stock solution of cysteine hydrochloride was made up by dissolving 157.5 mg. of cysteine hydrochloride in 10 ml. of 5M hydrochloric acid, and diluting to 100 ml. to make the solution 0.01M. It was

stored in the cold and no longer used after 5 or 6 hours. To standardize the solution the hydrochloric acid was neutralized by adding standard sodium hydroxide (using congo red as an indicator) and the neutral solution titrated with 0.01M potassium ferricyanide until a distinct yellow color was produced.(32).

Urea: For the denaturation of egg albumin and the production of sulfhydryl groups both Anson (24) and Mirsky (25) used solutions 8.3M in respect to urea. In his experiments with edestin and egg albumin, Burk (34) reported the minimal effective concentration for urea in aqueous solutions as 6.06M for 2% egg albumin, and 1.5M for 2% edestin solutions. In view of these figures, and also in view of the fact that at 1.67M sodium chloride concentration there is inhibition of sulfhydryl production below approximately 2.8M urea concentration for edestin (34), the urea concentration for the production of sulfhydryl groups in squash seed globulin was arbitrarily set at 4M. This value is loosely based on the assumption that squash seed globulin and edestin are similar in this respect.

Guanidine Hydrochloride: The denaturant guanidine hydrochloride was prepared from guanidine carbonate as directed by Anson (24), dried under vacuum, and kept in an air-tight container until used. Anson used between 1.4 and 2.4 gm. of guanidine hydrochloride per ml. of 2% egg albumin. An excess of guanidine hydrochloride was used in the

work on squash seed globulin in order to ensure denaturation.

Sodium Lauryl Sulfate: To take the place of Duponol PC, a denaturant used by Anson (24) and by Mirsky (25), a 10% solution of sodium lauryl sulfate was used. Duponol PC is a commercial product containing a mixture of C_{10} - C_{18} compounds of the series $CH_3(CH_2)_n \cdot CH_2OSO_3Na$. An excess of this denaturant was also used on squash seed globulin.

Hydrochloric Acid: Anson (24) carried out the denaturation of egg albumin with urea in acid medium, because it enhanced sulfhydryl production. Burk (34) confirmed this procedure but noticed that for lactalbumin a hydrochloric acid medium inhibited sulfhydryl production at low urea concentrations. Burk gave no figures for edestin with hydrochloric acid. According to Bawden and Pirie (35) urea can shift the pK values of proteins in solution. They also noted that the pH of protein solutions would increase 0.8 to 1.0 units on the addition of urea. In alkaline medium, other groups than sulfhydryl may reduce ferricyanide (25). In view of these facts the liberation of sulfhydryl groups from squash seed globulin was carried out in neutral or in slightly acid mediums.

Buffer Solutions: Buffer solutions were used in order to standardize the pH of experimental solutions. A M phosphate buffer pH 7.0 was made up by mixing equal parts of M K_2HPO_4 and M KH_2PO_4 solutions (25). A M phosphate buffer pH 6.4

was prepared by mixing equal parts of M Na_2HPO_4 and M NaH_2PO_4 solutions (24). Both solutions of buffers were checked on the Beckman pH meter. Buffer solutions from pH 4.0 to 6.2 were made up using the potassium acid phthalate - sodium hydroxide system (36).

Potassium Ferricyanide: All standard ferricyanide solutions were made up daily from a 0.1M stock solution, and kept in the cold. To oxidize sulfhydryl groups to disulfide stoichiometrically, Anson (24) used 0.05 millimoles of ferricyanide per 0.001 millimoles of ferrocyanide produced. Mirsky (25) used 0.003 millimoles of ferricyanide per 0.001 millimoles of ferrocyanide produced by egg albumin. In the oxidation of cysteine hydrochloride, Anson (22) obtained best results using 0.002 millimoles of ferricyanide for every 0.001 millimoles of ferrocyanide. For this work, in order to ensure an excess of ferricyanide, 0.01 millimoles were used in each experimental solution to oxidize any sulfhydryl groups from squash seed globulin.

Potassium Ferrocyanide: Standard ferrocyanide solutions were prepared daily from a 0.1M stock solution of potassium ferrocyanide kept in the cold.

Ferric Sulfate: To develop the Prussian blue color from the ferrocyanide formed, a solution of ferric sulfate in gum arabic was made up according to Folin and Malmros (37). Gum arabic was substituted for gum ghatti, which was unavailable. The effect of the gum is to prevent the

precipitation of the Prussian blue.

Sulfuric Acid: M sulfuric acid was added after the reaction of ferricyanide with sulfhydryl in order to prevent any further ferricyanide reduction by non-sulfhydryl groups in the mixture.

C. PRELIMINARY EXPERIMENTS

1. Standardization with Ferrocyanide:

For the first part of this problem the Lumetron colorimeter was used in order to estimate Prussian blue from ferrocyanide produced by the oxidation of sulfhydryl groups. The Lumetron was standardized in terms of cysteine according to the procedures of Mason (32) and of Anson (22). From the 0.01M stock solution, standard solutions of cysteine hydrochloride were prepared containing from 0.0015 to 0.0005 millimoles of cysteine. To each was added sufficient standard sodium hydroxide to neutralize the hydrochloric acid present, 2 ml. of 0.001M ferricyanide, 4 drops of phosphate buffer pH 7.0, and the mixture kept in a water bath at 37° for 5 minutes. After the reaction with ferricyanide, to each solution was added 0.5 ml. of ferric sulfate solution and enough distilled water to bring the volume of the mixture to 10 ml. The mixture was allowed to stand 5 minutes before reading the percent transmission in the Lumetron colorimeter. Readings were taken using both the yellow-green (530 mu wavelength) and the red (650 mu) filters. Control solutions containing no cysteine

were also run. Prussian blue was formed and the percent transmission determined under the same conditions with standard solutions containing from 0.0015 to 0.0005 millimoles of ferrocyanide.

TABLE I

Standardization of cysteine hydrochloride solutions with ferrocyanide solutions of the same strength under the same conditions, using two wavelengths on the Lumetron colorimeter for reading percent transmission of the Prussian blue developed.

		Percent Transmission			
		530 mu		650 mu	
Cysteine mM	Ferrocy mM	Cysteine	Ferrocy	Cysteine	Ferrocy
0.0015	0.0015	29.5	34.0	9.0	7.0
0.0014	0.0014	33.0	36.5	12.0	9.0
0.0013	0.0013	31.5	40.5	16.0	10.5
0.0012	0.0012	40.0	42.5	16.5	13.0
0.0011	0.0011	49.5	45.0	30.0	16.5
0.0010	0.0010	50.0	49.5	26.0	20.0
0.0009	0.0009	53.5	54.0	32.5	29.5
0.0008	0.0008	60.0	59.0	36.5	34.5
0.0007	0.0007	64.0	71.0	48.0	48.0
0.0006	0.0006	71.0	76.5	61.0	56.0
0.0005	0.0005	76.5	78.0	68.0	60.5

The results in Table I indicate that the red filter (650 mu) is impractical for the measurement of color intensity. Using the yellow-green filter (530 mu), the results show conformity to the equation:
2 sulfhydryl + 2 ferricyanide = disulfide + 2 ferrocyanide
only around a concentration of 0.001 millimoles of cysteine. Furthermore, the values for each determination varied as much as 4% transmission in four consecutive readings. Although the method appeared to be substantiated for a certain concentration of cysteine by this evidence, the degree of possible error was too high to warrant further use of the Lumetron colorimeter for purposes of estimating the Prussian blue color. Moreover, in view of subsequent work, the need of standardization curves for more precise determinations of the sulfhydryl groups from squash seed globulin was not yet indicated.

2. Oxidation of Cysteine Hydrochloride:

From the results of the above work it was decided that preliminary experiments with cysteine hydrochloride to determine the effect of reagents on the oxidation of sulfhydryl groups should be carried out at a concentration of 0.001 millimoles of cysteine. The procedure of Anson (24) for the production of sulfhydryl groups in egg albumin with urea was followed as closely as possible, with those modifications presumed necessary for the production of sulfhydryl

groups in squash seed globulin.

The procedure outlined below was considered suitable for cysteine determinations under these conditions. To 0.5 ml. of 0.002M cysteine hydrochloride solution were added 2 ml. of 25% sodium chloride solution and 2.5 ml. of 8M urea solution, thus making an acid solution, 10% in respect to sodium chloride and 4M in respect to urea. The mixture was held at 37° in a water bath for 5 minutes then 0.5 ml. of 0.02M ferricyanide solution were added. The ferricyanide was allowed to react for one minute at 37°, then 5 ml. of 0.0979M sodium hydroxide and 4 drops of phosphate buffer pH 7.0 were added to bring the pH to neutrality. After one minute 0.5 ml. of M sulfuric acid, 2.3 ml. of distilled water, and 1.0 ml. of ferric sulfate were added, bringing the total volume of the mixture to 10 ml. Thirty minutes after the addition of ferric sulfate the Prussian blue developed was read in values of percent transmission in the Junior Coleman photometer at 530 mu wavelength.

The junior Coleman photometer was considered more accurate for this type of work than the Lumetron colorimeter because of its narrower band width -- 35 mu. The above procedure deviated from that of Anson (24) in that the oxidation of sulfhydryl groups was carried out in 10% sodium chloride concentration, 4M urea concentration, and in an acidic medium. This acid medium was held for one minute before neutralization was effected. In those

solutions in which the sulfhydryl oxidation was carried out in neutral medium, very little Prussian blue was developed. The presence of 10% sodium chloride in contact with urea may account for the rise of the pH of the mixture above neutrality, which in turn would prevent the oxidation of sulfhydryl groups. The ability of urea to raise the pH of solutions was suggested by Bawden and Pirie (35).

To establish a relationship between intensity of color and quantity of ferrocyanide, Prussian blue was formed in solutions containing known amounts of ferrocyanide under precisely the same conditions as when cysteine hydrochloride was used. To 0.5 ml. of 0.002M potassium ferrocyanide solution were added the same reagents as above, and the results compared with those of the above procedure in Table II.

In order to observe the effect on the development of Prussian blue of the additional reagents, namely sodium chloride, urea, ferricyanide, sodium hydroxide, and sulfuric acid, used in the method already proposed for the liberation of sulfhydryl groups, the following procedure was adopted. Only the minimal reagents necessary to develop the Prussian blue from ferrocyanide solutions were employed. To 1 ml. of 0.001M potassium ferrocyanide solutions were added 0.2 ml. of phosphate buffer pH 7.0, 0.5 ml. of ferric sulfate, and enough water to bring the volume of the mixture to 10 ml. The Prussian blue was

estimated in the Junior Coleman photometer 30 minutes after adding the ferric sulfate.

TABLE II

Effect of the reagents on the oxidation of sulfhydryl groups and the development of the Prussian blue color in the proposed method for the liberation of sulfhydryl groups from squash seed globulin.

Added Cysteine	Added Ferrocy.	Reagents to form Prussian blue	Percent Transmission
1 mM	--	complete	51.5
--	1 mM	complete	53.0
--	1 mM	minimal	39.5

The results in Table II show that the complete reagents necessary to liberate sulfhydryl groups from squash seed globulin in urea hindered the development of Prussian blue, but did not interfere to any great extent in the oxidation of sulfhydryl groups. However, thirty minutes after the addition of ferric sulfate, the Prussian blue started to precipitate from all solutions. Up to this point the intensity gradually increased, the greater part of the color being formed at the end of fifteen minutes.

3. Liberation of Sulfhydryl Groups from Egg Albumin:

The preliminary experiments with cysteine proved essentially that any sulfhydryl groups liberated from squash seed globulin could be oxidized by ferricyanide, and Prussian blue developed using a modified method. This method was tried out on a 1.0% egg albumin solution 10% in respect to sodium chloride, prepared according to Anson's method (24). The same procedure was followed in detail, using 1 ml. of the egg albumin solution and 4 ml. of 10% sodium chloride solution to bring the total volume of the protein solution to 5 ml., and the total content of egg albumin to 10 mg. To this was added 2 drops of M hydrochloric acid, 2.6 ml. of 12M urea solution, and the mixture held at 37° for 30 minutes in a water bath. To oxidize sulfhydryl groups, 0.5 ml. of 0.02M ferricyanide was added and the reaction allowed to proceed for 5 minutes. The mixture was neutralized by adding 2 drops of M sodium hydroxide and 4 drops of phosphate buffer pH 7.0. After one minute oxidation was stopped by adding 0.5 ml. of M sulfuric acid. The precipitated protein was filtered off and 1 ml. of ferric sulfate solution added to the filtrate, bringing the total volume of the solution to 10 ml. The Prussian blue developed was read in 30 minutes as percentage transmission in the Junior Coleman photometer at 530 mu wavelength. A control solution containing no egg albumin was used to correct for the color contributed

by the reagents.

The Prussian blue developed in the filtrate showed 58.0% transmission in an average of triplicate runs. Anson (24) found that 0.001 millimoles of ferrocyanide were formed when ferricyanide was reduced by 10 mg. of denatured egg albumin in urea solution. In view of this value and the results of previous experiments with ferrocyanide in this problem, the value 58.0% is in close agreement with the work on cysteine hydrochloride. The higher transmission may be due to the lower concentration of urea used in this method, or to the presence of sodium chloride, both of which differ from the reagents used in Anson's procedures. However, the chief purpose of this preliminary experiment was to point out that the modified method, for the liberation of sulfhydryl groups in squash seed globulin, was valid for a protein that has been known to liberate sulfhydryl groups in urea solution.

D. EXPERIMENTS ON SQUASH SEED GLOBULIN

1. Urea Denaturation:

The proposed method was carried out on a solution containing 11.61 mg. of squash seed globulin (Protein I) per ml., and 10% in respect to sodium chloride. On the basis of a cysteine content of 0.51% for squash seed globulin (11), 5 ml. of this solution would contain 0.0024 millimoles of cysteine. The addition of hydrochloric acid

to the protein solution produced a slight turbidity that did not change after 30 minutes in 4M urea solution at 37°. The addition of ferricyanide caused an increase in turbidity; this condition persisted after neutralization of the mixture. A white flocculent precipitate was formed on adding sulfuric acid, which, after filtering, left a clear, light yellow solution. The average transmission of four identical runs was 98%. The same results were obtained with a solution containing 3.79 mg. of squash seed globulin (Protein II) per ml., and 10% in respect to sodium chloride. From this evidence it would appear that no sulfhydryl groups were produced in an exposed, active position by this method.

In the following experiments the different factors and reagents involved in this method were changed and revised in an effort to liberate sulfhydryl groups from the globulin. The results of each change in procedure were taken into consideration in the ensuing experiments.

To prevent the immediate precipitation of the globulin a procedure was tried involving the omission of hydrochloric acid. Buffer solutions of pH's 7.0, 6.4, 6.0, 5.6, and 5.2 were used in order to establish different levels of pH for sulfhydryl liberation and oxidation. In preliminary experiments with Protein I it was found that below a pH of 5.2 buffer solutions caused a flocculation of the protein. To 5 ml. of the protein solution were added 2.7

ml. of 12 M urea solution and 6 drops of buffer solution. After holding the mixture at 37° for 30 minutes, 0.5 ml. of 0.02M ferricyanide was added and any reaction allowed to proceed for 5 minutes. After adding 0.5 ml. of M sulfuric acid, the precipitate was filtered off and 1 ml. of ferric sulfate added to bring the total volume of the mixture to 10 ml. After 30 minutes no Prussian blue was formed in any of the solutions, although precipitation of the flocculent protein was prevented at all levels of pH until sulfuric acid was added. Ferric sulfate was poured over the residues of protein on the filter paper from each mixture; but after 60 minutes no color was produced, showing that no ferrocyanide existed in an occluded form on the precipitated protein.

Although the preceding experiment showed no evidence of ferricyanide oxidation of sulfhydryl groups, there remained the question of whether the protein was denatured by urea while it was in the unprecipitated form. To investigate this question qualitatively, Protein I was made 4M in respect to urea and held for 15 minutes at 37° in a water bath. To the resulting clear solution was added 4 parts by volume of water in an attempt to precipitate the crystalline globulin. A gummy, amorphous protein precipitate was formed on dilution that would not go back into solution on addition of an excess of 10% sodium chloride, which would ordinarily effect solution. The same results

were obtained with solutions of guanidine hydrochloride and sodium lauryl sulfate. When hydrochloric acid was added to the unprecipitated protein solutions after 15 minutes contact with these denaturants, the resulting precipitates were less flocculent than without denaturants present.

With 5 ml. of Protein I solution the foregoing procedure was carried out at pH 5.6 with an increase from 30 minutes to 2 hours in the time allowed for sulfhydryl liberation in urea solution. A slight turbidity was noticed in each solution at the end of 2 hours, however, no Prussian blue was developed.

In an attempt to eliminate any protein precipitation and to determine the effect of having all reagents present in the solution at the same time, the following procedure was employed. To 5 ml. of Protein I were added 2.7 ml. of 12M urea solution and 6 drops of buffer solution pH 5.6. The mixture was held at 37° for 2 hours, then 0.5 ml. of 0.02M ferricyanide added. After 5 minutes 1 ml. of specially prepared neutral ferric sulfate solution was added and the mixture allowed to stand 30 minutes. Although no precipitate was formed, no Prussian blue was developed.

The effect of an excess of protein was studied by adding 50 mg. of the dried squash seed globulin to each of three test tubes. To each was added 2 ml. of 25% sodium chloride solution, 1 gm. of urea, 0.5 ml. of M hydrochloric

acid, 0.5 ml. of pH 5.0 buffer solution, and the mixture held at 37° for one hour. One ml. of 0.02M ferricyanide was added and the reaction allowed to proceed for 10 minutes. The precipitated protein from the first test tube was filtered off, the filtrate made to 9 ml. with water, and 1 ml. of ferric sulfate added. The precipitate was also removed from the second test tube and the filtrate brought up to neutrality by adding 0.5 ml. of M sodium hydroxide and 0.5 ml. of pH 7.0 phosphate buffer. This mixture was made to 9 ml. with water and 1 ml. of ferric sulfate added. To the third test tube was added 1 ml. of ferric sulfate, with no filtration of the precipitate. After one hour in urea and sodium chloride solutions, approximately one half of the protein in all test tubes had changed from the crystalline state to a flocculent, amorphous mass. Ten minutes after adding ferric sulfate no color was evident in any solution. At the end of one hour the precipitated protein in the third test tube showed evidence of Prussian blue occluded to it, while the other two solutions exhibited no color. On acidifying the filtrate from the second test tube a deep Prussian blue color was developed.

To investigate the possibility that on long time exposure of squash seed globulin to urea there was caused a liberation of sulfhydryl groups in the preceding experiment, the procedure was repeated with an increase from

1 to 15 hours in the time of holding the protein solution at 37° in a water bath. No Prussian blue was developed in any solution 15 minutes after adding ferric sulfate. The third test tube, containing the precipitated protein, again gave evidence of Prussian blue occluded to the precipitate after standing one hour.

The effect of sodium chloride on the reactions of the above procedure was studied by omitting the addition of 2 ml. of 25% sodium chloride solution, and reducing the holding time at 37° to 2 hours. No protein appeared to go into solution, but rather the crystalline globulin turned into a partially gummy, denatured mass. The results were the same as in the foregoing work, including the appearance of Prussian blue formation after long time standing of the solution containing the precipitated protein.

2. Hydrochloric Acid Denaturation:

In preliminary experiments with hydrochloric acid as a denaturant it was found that the flocculent precipitate produced when hydrochloric acid was added to a solution of squash seed globulin in 10% sodium chloride could be dissolved by adding sodium hydroxide immediately after precipitation. However, after 15 minutes standing in acid medium, no dissolution of the precipitated protein could be effected by adding even concentrated sodium hydroxide.

The proposed method for the liberation of sulfhydryl groups from squash seed globulin by urea was carried out on 5 ml of Protein I, using 2.6 ml. of 0.01M hydrochloric acid in place of urea. No Prussian blue was formed in any of several runs made.

The same procedure was repeated with the ferricyanide added to the protein solution before holding the mixture at 37° for 30 minutes. No change in results was shown.

The effect of an excess of protein was studied by adding 4 drops of M hydrochloric acid and 2 ml. of water to 50 mg. of the dried protein. The mixture was held at 37° for 2 hours and 0.5 ml. of 0.02M ferricyanide added. After 5 minutes 1 ml. of ferric sulfate was added and the color development estimated after 30 minutes. A slight amount of green color was noticed on the precipitated protein. As in the experiments with urea, part of this protein was in the form of a flocculent precipitate, the remainder a gummy mass after denaturation.

3. Sodium Lauryl Sulfate Denaturation:

A 10% solution of sodium lauryl sulfate was employed as a denaturing agent for squash seed globulin, with the omission of hydrochloric acid from the procedure in order to keep the protein in solution in the presence of ferricyanide. To 5 ml. of Protein III were added 6 drops of buffer solution pH 6.0, 1 ml. of 10% sodium lauryl sulfate,

and the mixture held at 37° for 15 minutes, followed by the addition of 0.5 ml. of 0.02M ferricyanide solution. After 2 minutes the mixture was made to 9 ml. with water and 1 ml. of ferric sulfate added. The protein precipitated on the addition of ferric sulfate, although the sodium lauryl sulfate content was increased in an effort to prevent flocculation of the protein. After a period of 30 minutes no Prussian blue was noticed in any solution.

The foregoing procedure was used on 50 mg. of the dried globulin with no sodium chloride present, and the denaturation allowed to proceed for 2 hours. No Prussian blue was developed. When 4 drops of hydrochloric acid were added to the mixtures before holding at 37°, the precipitated protein became more flocculent, but no color was produced. The same results were obtained on increasing the holding time from 2 to 15 hours.

4. Guanidine Hydrochloride Denaturation:

Guanidine hydrochloride was employed in the solid form as a denaturant on squash seed globulin because of the instability of its aqueous solutions. The denaturation was carried out at different levels of pH, namely 7.0, 6.4, 5.0, and 4.0, using identical procedures for solutions containing 50 mg. of the dried globulin, and for solutions containing 4 ml. of Protein III. To each protein solution were added 0.2 gm. of guanidine hydrochloride, 0.5 ml. of buffer solution, 2 ml. of water, and the mixtures held at

37° for 60 minutes. One ml. of 0.02M ferricyanide was added and the mixtures allowed to stand 10 minutes before adding 1 ml. of ferric sulfate. Protein precipitates formed in the solutions containing Protein III when ferric sulfate was added, but no color was formed. The solid protein did not appear to dissolve to any extent; however, Prussian blue began to form on the precipitate after 30 minutes standing.

The above work was repeated with the ferricyanide in the presence of the protein during denaturation. Although the amount of Prussian blue occluded to each precipitate was slightly increased, this color did not appear until the solutions had been standing 30 minutes.

5. Heat Denaturation:

To study the effect of heat denaturation on the liberation of sulfhydryl groups from squash seed globulin, 4 ml. of Protein III were heated for 30 minutes in a hot water bath at 100° in the presence and in the absence of 1 ml. of 0.01M ferricyanide. Different levels of pH were maintained during denaturation by adding buffer solutions of pH's 7.0, 6.4, 5.0, and 4.0. To all solutions were added 1 ml. of 0.01M ferricyanide and 1 ml. of ferric sulfate. No color was produced until the solutions had stood for 30 minutes. In all solutions a precipitate was formed after heating which was less flocculent than that produced by hydrochloric acid. The supernatant liquid above each

precipitate was found to be quite turbid, and from it a flocculent precipitate could be formed by adding 2 drops of M hydrochloric acid.

E. NITROPRUSSIDE TESTS

The reaction of squash seed globulin to the nitroprusside test was taken as a criterion of the validity of the ferricyanide method used in this problem. The results of this test, using Protein III, were compared with those of a 1.5% solution of egg albumin, 10% in respect to sodium chloride, and a 0.001M solution of cysteine hydrochloride under the same conditions. To 4 ml. of the protein or amino acid solution were added 0.2 ml. of M hydrochloric acid and 1 gm. of urea, and the mixture held at 37° for 15 minutes. On completion of the denaturation 4 drops of 5% sodium nitroprusside solution and 2 drops of concentrated ammonium hydroxide were added. The egg albumin and cysteine hydrochloride solutions immediately turned reddish purple, indicating positive tests for sulfhydryl groups. The squash seed globulin solution remained a yellow-brown in color. Using the same method of denaturation, both egg albumin and cysteine hydrochloride gave positive tests for ferricyanide while squash seed globulin showed a negative test. In both tests the color developed in aqueous solutions of egg albumin was greater than that developed in sodium chloride solutions,

in which a slight precipitation was noticed. However, the alternative tests carried out without the addition of hydrochloric acid, and thus without the precipitation of either squash seed globulin or of egg albumin, gave identical results as when a precipitate was present.

F. IODINE TITRATION OF SQUASH SEED GLOBULIN

An attempt was made to duplicate the work of Hess and Sullivan (11) on the cysteine content of native squash seed globulin. By direct titration with iodine according to the Okuda method for cysteine these workers found a cysteine content of 0.51% in squash seed globulin.

A protein solution containing 11.61 mg. of globulin per ml. in 10% sodium chloride solution was made up from the dried material prepared in 1945. Ten ml. of the protein solution were made 2% with respect to hydrochloric acid by adding 10 ml. of 4% acid. Five ml. of 4% hydrochloric acid and 5 ml. of 5% aqueous potassium iodide were added. The solution was cooled to 20° and titrated to a permanent yellow color with M/600 potassium iodate (in 2% HCl) that had been standardized against cysteine hydrochloride similarly treated.

The globulin precipitated in an amorphous, flocculent mass as soon as the first drop of acid was added. The end-point of the titration was reached with the first drop of

iodate. The end-point was also reached with the first drop when M/1200 potassium iodate was used, although theoretically 0.75 ml. of iodate should have been used on the basis of a cysteine content of 0.51% in squash seed globulin. The addition of 2 ml. of 1% starch solution in order to provide a clearer end-point, gave the same results. In further titrations 10 ml. of 2M glucose in 4% hydrochloric acid solution were used in order to make the protein 2% with respect to hydrochloric acid but no difference in results was evident.

An effort was made to prevent precipitation of the globulin by changing the sodium chloride concentration to 4%. At 20° precipitation of the crystalline globulin is just prevented in this concentration of sodium chloride. It was found possible to add more acid to this solution before precipitation of the amorphous globulin was effected. However, before the concentration of acid reached 2% the protein was again precipitated as a flocculent mass.

To keep the protein in solution it was decided to omit all hydrochloric acid additions except that present in the potassium iodate. To 10 ml. of squash seed globulin solution in 10% sodium chloride were added 5 ml. of aqueous potassium iodide, 10 ml. of phosphate buffer pH 6.4, and 2 ml. of 1% starch solution. Four ml. of M/1200 iodate were used in the titration. The same value was obtained when no starch was added, but in both cases the end-point of

the titration was quite vague. The value obtained represents a cysteine content of 2.72% for squash seed globulin. However, on the addition of the required amount of hydrochloric acid to these titrations, the depth of the color immediately produced indicated that the end-point of the titration had been reached and exceeded to some extent.

THE EFFECT OF SUGARS ON THE COAGULATION OF SQUASH SEED GLOBULIN

A. Coagulation by Hydrochloric Acid:

To study the effect of sugars on the coagulation of squash seed globulin by hydrochloric acid, the following procedure was drawn up using Protein III, containing 1.67 mg. of nitrogen per ml., and in a solution 10% in respect to sodium chloride. To 4.5 ml. of the protein solution were added 5 ml. of sugar solution and the mixture held at 37° in a water bath for 15 minutes in order to allow any combinations between sugar and protein to take place. Four sugar solutions were used: 2M D-glucose, M D-glucose, M D-galactose, and M D-levulose, all of which were made 10% in respect to sodium chloride in order to prevent protein precipitation as crystals. All sugars were of Pfanstiehl C. P. anhydrous quality from the following lots: D(+)glucose, ---. #1655; D(-)levulose, ---. # 1675; D(+)galactose, ---. #1601. Coagulation of the protein was effected by adding 0.5 ml. of M hydrochloric acid and the solution allowed to stand 2 minutes at 37°. The coagulated protein was removed by filtration, using ordinary qualitative filter paper, and the amount of coagulation was determined by analyzing the filtrate for nitrogen by Microkjeldahl method (33). All values in

Table III represent the average nitrogen value of triplicate determinations. A control solution containing no protein was run on each sugar solution and a correction made for nitrogen from this source. The pH of all solutions was found to be 1.2 on the Beckman pH meter.

TABLE III

Effect of sugars on the coagulation of squash seed globulin by hydrochloric acid, using 2 ml. of the filtrate for nitrogen determinations. Original nitrogen in 2 ml. of the filtrate = 1.50 mg.

Sugar Conc.	Mg. N in 2 ml. of Filtrate	% of original N in Filtrate
M D-glucose	0.08	5.3
0.5M D-glucose	0.00	0.0
No D-glucose	0.05	3.3
M D-galactose	0.05	3.3
No D-galactose	0.05	3.3
M D-levulose	0.00	0.0
No D-levulose	0.05	3.3

B. Coagulation by Heat:

Protein III was again used to study the effect of the same sugars at the same concentrations on the heat coagulation of squash seed globulin. A phthalate - sodium hydroxide buffer of pH 6.0 was used to establish the pH of the solutions. To 4.5 ml. of the protein solution were added 5 ml. of sugar solution, 0.5 ml. of buffer solution pH 6.0, and the mixture held for 30 minutes at 80° in a water bath. The coagulated protein was filtered off, using quantitative filter paper, and a 2 ml. portion of the filtrate used for nitrogen determinations by Microkjeldahl method (33). Determinations were run in triplicate and correction was made for the nitrogen contributed by the sugar and by the reagents. The results are given in Table IV.

The coagulate from heat denaturation differed from that formed by denaturation with hydrochloric acid in that the former was less flocculent, and left a turbid filtrate that could neither be filtered off by quantitative filter paper, nor centrifuged off in 60 minutes.

TABLE IV

Effect of sugars on the coagulation of squash seed globulin by heating 30 minutes at 80°, and at a pH of 6.0. Original nitrogen content of 2 ml. of the filtrate used in nitrogen determinations = 1.50 mg.

Sugar Conc.	Mg. N in 2 ml. of filtrate	% of original N in filtrate
M D-glucose	1.13	75.3
0.5M D-glucose	1.09	72.7
No D-glucose	1.16	77.3
M D-galactose	1.03	68.7
No D-galactose	1.16	77.3
M D-levulose	1.21	80.7
No D-levulose	1.16	77.3

From Table IV it was concluded that at 80° coagulation was not sufficiently complete to study the effect of the sugars on coagulation of the globulin. Furthermore, the presence of the buffer solution was thought to have an effect on the coagulation, and therefore was omitted in the next experiment, in which the procedure is essentially the same as the preceding one. Centrifugation of the coagulated protein mixture was substituted for filtration with quantitative paper in order to eliminate any error from adsorption on the paper, although the filtrates in both cases were almost the same in nitrogen content as revealed by Microkjeldahl determinations on identical solutions. To 5 ml. of the Protein III solution were added 5 ml. of the sugar solution, and the mixture placed in a water bath. The bath was allowed to heat up slowly from 25°, taking 5 minutes to reach boiling temperature, and held at 100° for 15 minutes. According to Osborne (1) this slow rise of temperature enhances the coagulation of the globulin at lower temperatures during a given period of time. The mixture was centrifuged for 5 minutes after coagulation, and 2 ml. of the supernatant liquid used for nitrogen determinations by Microkjeldahl method. The results shown in Table V represent the average of triplicate determinations at each sugar concentration.

TABLE V

Effect of sugars on the coagulation of squash seed globulin by heating at 100° for 15 minutes. Original nitrogen content in 2 ml. of the filtrate used for nitrogen determinations = 1.67 mg.

Sugar Conc.	Mg. N in 2 ml. of Filtrate	% of original N in filtrate
M D-glucose	1.08	64.7
0.5M D-glucose	0.99	59.3
No D-glucose	1.04	62.3
M D-galactose	0.91	54.5
0.5M D-galactose	1.08	64.7
No D-galactose	1.04	62.3
M D-levulose	0.88	52.7
0.5M D-levulose	0.87	52.1
No D-levulose	1.04	62.3

Because of an apparent difference in the turbidity of the supernatant liquids from the solutions used in Table V, the percentage transmission of each solution was determined in the Junior Coleman photometer at 550 mu wavelength. To complete Table VI, readings were also made on 0.25M and 0.1M solutions of the same sugars, as well as solutions containing no sugar, under identical procedures of denaturation and centrifugation. The instrument was set to read 100% transmission with a control solution containing 10 ml. of 10% sodium chloride solution.

TABLE VI

Percentage transmission of the supernatants from squash seed globulin solutions containing 9.79 mg. of protein per ml., heated at 100° for 15 minutes. Readings made on the Junior Coleman photometer at 550 mu wavelength. Total volume of each solution = 10 ml.

	CONCENTRATIONS				
Sugar	1 M	0.5 M	0.25 M	0.1 M	No Sugar
D-glucose	38.5	24.2	15.3	7.0	7.5
D-galactose	51.3	42.0	11.8	8.5	7.5
D-levulose	37.5	19.5	16.0	7.5	7.5

DISCUSSION

With respect to the methods employed in this problem for the liberation and estimation of sulfhydryl groups, the protein squash seed globulin did not produce sulfhydryl groups in an active, exposed form on denaturation by urea, guanidine hydrochloride, sodium lauryl sulfate, hydrochloric acid, or by heat. Neither was there evidence of the presence of sulfhydryl groups in the native protein when the same methods of estimation were used. According to the protein classifications set forth by Neurath, Greenstein, Putnam, and Erickson (16) this characteristic would put squash seed globulin in that class of proteins, such as amandin and insulin, which neither in the native state nor after treatment with concentrated guanidine hydrochloride give tests for sulfhydryl. Although there is a similarity between squash seed globulin and amandin in that they are both seed globulins, two other seed globulins, edestin and excelsin, belong to that class of proteins which in the native state do not give tests for sulfhydryl, but do so after treatment with concentrated guanidine hydrochloride (16). However, as Gortner points out (38), the classification of proteins as globulins represents a very poorly defined group.

Hess and Sullivan (11) concluded from their work on squash seed globulin that cysteine complexes were present

in the native protein, and substantiated their claim by reporting values for the iodine titration of the native protein according to the Okuda method for cysteine. An attempt to duplicate these values in the problem failed because of the persistent appearance of a flocculent protein precipitate on the addition of the acid necessary to carry out the titration. Hess and Sullivan stated that aqueous or dilute salt solutions of the proteins were used in their experiments, and from this it was assumed that a sodium chloride solution was used, since the protein is practically insoluble in water. The sodium chloride content of the solutions was varied over a wide range, but with no prevention of precipitation on the addition of hydrochloric acid. A high value for cysteine was obtained by omitting the required acid, but experiments with cysteine hydrochloride pointed out that the conditions of the titration must be followed closely in order to secure accurate results.

If cysteine complexes are present in squash seed globulin, then this protein can be placed in the same classification as edestin and excelsin, in which it was assumed that the cystine-cysteine sulfur was composed of cystine sulfur plus cysteine sulfur (16). This grouping is not in agreement with the previous grouping of squash seed globulin with amandin and insulin, in which the cystine-cysteine sulfur is composed entirely of cystine

sulfur (16). It follows then that the methods of liberating and estimating sulfhydryl groups of squash seed globulin in this problem would be considered inadequate if the grouping with edestin and excelsin is accepted, unless the inherent properties of the protein are such that liberated sulfhydryl groups are not produced in an active form on denaturation with the above mentioned agents.

The method proposed in this work for the liberation and estimation of sulfhydryl groups from squash seed globulin was found to be effective for cysteine hydrochloride and for egg albumin solutions, but gave negative results on the globulin itself. The presence of precipitated protein during the exposure of the globulin to ferricyanide was thought to prevent the liberation of sulfhydryl groups. However, on elimination of this precipitate by omitting the addition of hydrochloric acid and using buffer solutions to establish different levels of acidity for sulfhydryl oxidation, no Prussian blue was developed. The time period of exposure of the protein to the denaturing agents was increased to two hours with no change in results.

The appearance of Prussian blue occluded to the precipitated protein in the experiments with excess protein in the solid state came as a result of long time standing of the precipitated protein in the presence of sodium chloride, urea, ferricyanide, and ferric sulfate. Since it takes only one minute for any reactive sulfhydryl groups

to reduce ferricyanide (24), and since the greater part of Prussian blue development is achieved in five minutes from the time of adding ferric sulfate, this subsequent color was presumed to be the result of ferricyanide reduction by other than sulfhydryl groups. Furthermore, an increase in the time of exposure to the denaturing agent to 15 hours produced no difference in the length of time elapsed before the appearance of Prussian blue on the precipitate, which showed that sulfhydryl groups were not liberated by long time standing of the protein in contact with the denaturant.

In those solutions in which acidification of the final mixture brought about a development of Prussian blue, the color was attributed to the existence of protein and ferricyanide in a neutral or slightly alkaline medium, caused by the presence of urea in the solution (35), which would enhance the ability of non-sulfhydryl groups on the protein to reduce ferricyanide (25). In order to confirm this reduction of ferricyanide by non-sulfhydryl groups, preliminary experiments were run on squash seed globulin using hydrochloric acid as a denaturant. On completion of the denaturation of the protein, ferricyanide was added and the mixture brought up to neutrality by the addition of M sodium hydroxide and phosphate buffer solution of pH 7.0. Four drops of M sodium hydroxide were added and the mixture held at room temperature for 5 minutes before acidifying with 6 drops of M hydrochloric acid and adding ferric

sulfate. The presence of ferricyanide in contact with the protein in alkaline medium produced a deep Prussian blue color on acidification of the final mixture.

When sodium chloride was omitted from the procedure by adding the desired reagents to the dried protein, a similar appearance of Prussian blue occluded to the precipitated protein was noticed after long time standing of the final mixture. If any of the protein had gone into solution, this would indicate that the presence of sodium chloride alone did not inhibit the production of sulfhydryl groups from squash seed globulin. On the other hand, if none of the protein had dissolved, the presence of Prussian blue in the mixture could definitely be ascribed to reduction of ferricyanide by other than sulfhydryl groups. Lastly, the preliminary experiments with cysteine hydrochloride showed that the presence of sodium chloride did not inhibit the oxidation of active sulfhydryl groups by ferricyanide.

The comparative results of the nitroprusside test on squash seed globulin, egg albumin, and cysteine hydrochloride confirmed those of the ferricyanide reaction in that no sulfhydryl groups were liberated from squash seed globulin when denatured by the above mentioned agents.

In the preliminary experiments on the denaturation of squash seed globulin by urea, guanidine hydrochloride, and sodium lauryl sulfate, the criterion of denaturation

was taken as the appearance of a gummy, insoluble precipitate on dilution of the denatured protein solution with water, and the subsequent failure of this precipitate to dissolve in 10% sodium chloride solution. This state of denaturation was reached by holding the sodium chloride solutions of the protein in contact with the denaturant for 15 minutes. No investigation was completed on the minimal concentration of denaturant necessary to effect denaturation. In the case of hydrochloric acid, the failure of the acid precipitated protein to dissolve in alkaline medium was taken as the criterion of denaturation. This condition was attained by holding the acid precipitated protein at room temperature for approximately 15 minutes. The appearance of a gummy coagulate when squash seed globulin solutions were heated at 100° for 15 minutes was considered a reaction of denaturation.

D-glucose, D-galactose, and D-levulose appeared to have little or no effect on the coagulation of squash seed globulin by hydrochloric acid, when the degree of coagulation was indicated by the relative amount of nitrogen remaining in the filtrate. The results in Table III are apt to show an inhibition of protein coagulation in the presence of M glucose and an increase in the presence of 0.5M glucose. However, in view of the completeness of coagulation and the resulting low nitrogen content of the filtrates, these results are misleading. A difference of 0.03 mg. in the amount of nitrogen in 2 ml. of the

filtrate corresponds to an acid titration value of 0.10 ml. This value is quite insignificant when consideration is given to the amount of acid necessary to correct for the reagents themselves and for the nitrogen contributed by the sugar. These amounts were respectively 0.20 and 0.21 ml. Furthermore, the degree of experimental error is greatly increased by the large amounts of sugar to be digested in the Microkjeldahl method of determining nitrogen in the filtrates.

The effect of D-glucose, D-galactose, and D-levulose on the heat coagulation of squash seed globulin at 80° and at a pH of 6.0 appeared to be small or non-existent. The use of qualitative filter paper for the filtration of the coagulated protein solution in this experiment and in the experiment with hydrochloric acid coagulation might have accounted for a certain amount of error in results. In the experiment with heat coagulation at 100°, any error due to adsorption of the protein on the filter paper or to incomplete filtration was eliminated by centrifugation of the coagulated mixture.

The effect of D-glucose, D-galactose, and D-levulose on the heat coagulation of squash seed globulin at 100° in an unbuffered solution was found to be the same as at 80°. The variation in percentages of original nitrogen in the supernatant solutions after coagulation was sufficiently small to be attributed to the experimental error

encountered in the Microkjeldahl method of determining nitrogen. However, the differences in percentage transmission of the filtrates from various concentrations of each sugar indicated that the presence of sugars during the heat coagulation of squash seed globulin tended to keep the protein in a more dispersed state. This inference was based on the observation that the amount of nitrogen in each supernatant solution, as determined by Microkjeldahl analysis, was approximately the same.

The apparent anomaly between the results obtained in the nitrogen determinations of the supernatant solution and those obtained by percentage transmission readings might be explained as due to the finite difference in the degree of dispersion of the protein as influenced by sugars.

CONCLUSIONS

1. Sulfhydryl groups, as estimated by the ferricyanide method, are not liberated from squash seed globulin in an active form by urea, guanidine hydrochloride, sodium lauryl sulfate, hydrochloric acid, or heat.

2. On the basis of solubility tests, squash seed globulin can be irreversibly denatured by urea, guanidine hydrochloride, sodium lauryl sulfate, hydrochloric acid, and heat.

3. D-glucose, D-galactose, and D-levulose have no effect on the coagulation of squash seed globulin by hydrochloric acid or by heat.

4. The degree of dispersion of squash seed globulin on heating in sodium chloride solution is intensified by the presence of D-glucose, D-galactose, and D-levulose.

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