Chemistry of the heat coagulation of proteins, no.1: heat coagulation of edestin from hemp seed
by Sami Wafa Dajani

A THESIS Submitted to the Graduate Ccmittee in partial fulfillment of the requirements for the Degree of Master of Science in Chemistry at Montana State College
Montana State University

Abstract:
1. No appreciable quantity of ammonia could be detected when edeatin was coagulated by heat, 2. When edestin which has been dissolved in ten per cent sodium chloride solution was boiled (boiling point 95° C.) for thirty minutes it was only partially coagulated, 3. Heat coagulation is not an instantaneous process.

CHEMISTRY OF THE HEAT COAGULATION OF PROTEINS

No. I

HEAT COAGULATION OF EDESTIN FROM HEMP SEED

by

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Part I

Introduction

Heat coagulation of protein substances is a phenomenon of frequent occurrence. It plays an important part in our daily life, in our industry and in our agriculture. The literature on the heat coagulation of proteins is very conflicting and investigators in this field even vary widely in their interpretation of the few experimental data which are considered definitely establish. The only point of agreement among such investigators is that heat coagulation of a protein occurs in two stages: (a) denaturation, and (b) agglutination, or the separation of the denatured protein in particulate form. Of these two phases, the one indicated by the term aggutination is most easily explained in terms of modern chemical formulation. Very diverse opinions on the other hand are held at present with respect to the essential chemistry of the denaturation phase.

Part II.

Review of the Literature on Heat Coagulation.

Heating solutions of mahy proteins causes, as the temperature rises, an irreversible change---the so called "heat coagulation"---to take place. Chick and Martin (1) believed that the heat coagulation of proteins consists of two distinct processes (a) "denaturation", or the reaction between protein and hot water, and (b) "agglutination", or the separation of the altered protein in particulate form. They point out that in acid solution agglutination occurs at a rate very greatly in excess of denaturation because if the protein is coagulated and the filtrate separated from the coagulum it does not show any change (formation of coagulum) if set aside for some time. The denaturation process, therefore, becomes the limiting factor and the rate of the heat coagulation of a protein can be studied without much complication. The heat plays only the part of accelerator. Chick and Martin showed that the temperature coefficient of heat coagulation is very high. The velocity constant increases nearly fifteen times for a ten degree rise in temperature between 60 and 70 degrees. The velocity constant is found from the equation

$$K = \frac{\log C_o - \log C_t}{t}$$

where C_o is the initial concentration of albumin and C_t is the concentration at time t. The velocity constant at 60° C. for

egg albumin is 0.0094 and at 70.4° C. it is 0.15 for the same material. However, when egg albumin is heated in alkaline solution agglutination does not occur, but may be induced by subsequent acidification or saturation with sodium chloride. They found that the reaction velocity varies considerably with different proteins and it also varies according to the acidity and the saline content of the solution. The presence of acid permits heat coagulation at a lower temperature than when acid is not present. If an acid were added in equal quantities to a solution of albumin, the first additions produce a small fall in coagulation temperature, but the acid effect becomes greater and greater with each addition, till a point is reached where the velocity of denaturation becomes so rapid as to make it difficult to determine the velocity constant. Thus the coagulation rate is not directly proportional to the hydrogen ion concentration. As, however, most of the acid added to a protein combines with protein molecules to form salts it may be that the whole or part of the effect of acid upon reaction rate is due to these protein salts reacting with water more rapidly than the protein itself reacts and that the more acid combined with the protein the more the coagulation rate is increased. The free acid in a solution of egg albumin diminishes, and even disappears as coagulation proceeds. The quantity of acid fixed by the coagulation of a definite quantity of protein is at first nearly proportional to the

concentration of free acid, but as the concentration increases the amount fixed falls more and more out of proportion. The phenomenon is probably one of adsorption inasmuch as washed coagulated protein adsorbs acid from solution. Thus Chick and Martin (1) found that egg albumin, precipitated by heating in nearly neutral solution, removed acid from 0.003 normal hydrochloric acid solution.

The denaturation rate of egg albumin by heat in alkaline solutions has been shown by Chick and Martin (1) to increase with increasing concentration of the hydroxyl ions exactly as was stated previously relative to the increase in the rate of heat coagulation in acid solution. In the neighborhood of the neutral point the change in the hydrogen or hydroxyl ion concentration is less effective in modifying the denaturation rate than in regions further removed. Increase in alkalinity from $10^{-3.8}$ normal to $10^{-2.7}$ was accompanied by an increase in average denaturation rate of over a hundred fold. They also showed that the hydroxyl ion is continuously removed as denaturation proceeds in alkaline solution in the same manner as in acid solution. In alkaline solution, however, agglutination does not occur due to the electric charge given to the particles. If the electric charge is neutralized and the protein becomes isoelectric agglutination occurs. In order to bring about agglutination of the protein which has been denatured by heat, the samples tested by Chick and Martin (1) were acidified by the addi-

tion of one tenth normal acetic acid in slight excess of the amount of alkali originally added. Enough pure sodium chloride was then added to saturate the solution. After standing for 24 hours, the coagulum on boiling was determined in a measured volume of the filtrate. This method was found to be useful in securing complete precipitation of the denatured protein in cases where the reaction was not correctly adjusted for most complete agglutination.

The progress of the reaction of protein denaturation by heat as ordinarily carried out conforms to no known simple law. Thus the reaction velocity does not remain proportional to the concentration of the residual protein, but departs more and more from the theoretical value as the experiment proceeds. This is due, according to Chick and Martin (1) to the progressive diminution in the alkalinity which is taking place in the solution during the progress of heat coagulation at constant temperature. The attempt was then made to keep the alkalinity constant during the process of denaturation by means of the presence of excess of solid magnesium oxide, a solution of which possesses a hydroxyl ion concentration of about 10^{-4} normal. When the hydroxyl ion concentration was thus maintained constant the denaturation rate of heat coagulation of egg albumin at constant temperature was found to follow a first order reaction. Thus if the concentration of the residual albumin in solution at any time is plotted against time a straight line is obtained. In the same

way, if the acidity is kept constant during the process of heat coagulation of egg albumin by the means of the presence of excess solid boracic acid, a saturated solution of which possesses a hydrogen ion concentration of $10^{-3.1}$ normal, coagulation proceeds as a reaction of the first order and the coagulation rate at any moment is proportional to the concentration of the albumin in solution. Chick and Martin (1) thus believed that heat coagulation is a first order reaction between protein and water, with temperature, salt concentration and hydrogen ion concentration functioning merely as accelerators.

As shown by Chick and Martin (1) heat coagulation does not occur in the absence of water below 120° C. These investigators heated egg albumin in the absence of moisture at 120° C. for five hours after which period of heating they found no evidence of denaturation as denoted by no change in solubility in water. The agglutination or the separation of the denatured protein in particulate form, is influenced greatly by the presence of neutral salts. In the case of egg white, agglutination of denatured protein is assisted by the presence of electrolytes and the hydrogen ion concentration. In the case of solutions of other proteins, however, agglutination is hindered by presence of salts.

Chick and Martin (1) pointed out that the coagulation temperature which has been almost universally regarded as a physical characteristic of the particular protein, is subject to

variations according to the conditions of the experiment; and, therefore, it is entirely misleading to regard this temperature as a constant characteristic of the protein unless the conditions of the experiment are specified. Thus the coagulation temperature of serum globulin was found to range from 72° - 75° centigrade. The variation apparently depends upon the rate of heating, on the concentration of salt, and on the concentration of the protein. A fourth factor which exerts a profound influence on the coagulation temperature is the addition of even minute amounts of acid or alkali. It was found in the case of serum albumin that neutralizing an alkaline solution to litmus lowered the coagulation temperature from 80° to 78° C., and that by the successive addition of small quantities of acid, it fell as low as 53° C.

Hycraft and Duggan (8) pointed out that the act of coagulation takes a certain time and that the temperature of coagulation rises as the concentration of the protein diminishes.

T. B. Robertson (14) believes that the phenomenon of heat coagulation is a phenomenon of dehydration and not of hydration as suggested by Chick and Martin (1). He points out that the base combining capacity of casein diminishes with the rise in temperature due to the fact that it undergoes some measure of polymerization which in turn is due to the dehydration of the end amino and carboxy groups. He believes that denaturation

corresponds to the dehydration of the protein molecule, and that agglutination corresponds to the simultaneous polymerization of these anhydrides.

Wu and Wu (16) claim to have shown that when egg albumin is coagulated by heat tyrosine splits off.

Mastin and Rees (9) repreated Wu and Wu's experiments but found no evidence that tyrosine is split off when albumin is coagulated by heat. The method they used in the estimation of the amount of tyrosine was the Weiss modification of the Millon's reaction which could be used to detect one part of tyrosine in 500,000 parts of solution.

PART III

Principles Governing the Selection of Material for Study of Heat Coagulation

Preparation and Properties of Edestin

My early work on the study of heat coagulation was done with crystalline egg albumin. Crystalline egg albumin was prepared in the usual way, recrystallized several times and was dialyzed until free from ammonium sulfate. Toluene was used as a preservative during the process of dialysis. Mastin and Rees (9) reported the fact that when egg albumin is heated for a period of 15 to 30 minutes at 60° or 70° C. 0.5% of the nitrogen present originally remains in solution. This nitrogen seems to decrease on further prolonged heating. The experimenter suggested that this nitrogen might arise from the ammonium sulfate which was used in the precipitation of albumin, and which was not removed by dialysis. They, however, did not attempt to prove it experimentally. My interest in the source of this nitrogen led me to choose a protein which could be extracted and purified without the use of nitrogenous substances. Edestin from hemp seed proved such a substance.

When dialyzed egg albumin was dissolved in water and heated, a minute amount of ammonia was eliminated. It was thought that the source of this ammonia was most probably ammonium sulfate which had been used in the precipitation of egg albumin. To test this supposition, it was planned to determine whether ammonia is given

off when a protein like edestin (in the extraction of which no nitrogenous substance need be used) was heated.

Edestin conforms strictly to the definition of globulin: it being insoluble in water but soluble in sodium chloride solution. Edestin has the useful property of being much less soluble in cold brine than in warm. This property makes it possible to obtain edestin of high purity.

Definition of Globulin: Globulins are defined as simple proteins soluble in neutral salt solutions of the salts of strong acids and bases, but are insoluble in water. They are usually heat coagulable.

Occurrence: Globulins occur in animals and plants. In the case of plants globulins are to be found in seeds and buds as storage material. In animals they occur in the blood.

The Animal and Plant Globulins: Globulins obtained from animal sources differ in certain properties from those obtained from plant sources. Animal globulins are precipitated by saturating their solutions with $MgSO_4$, but many of the vegetable globulins can not be thus precipitated. Animal globulins are precipitated by half saturation with ammonium sulfate but many plant globulins are precipitated at less or considerably more than half saturation. Saturation with Na_2SO_4 at 33° precipitates both animal and plant globulins.

Preparation of Plant Substance Before Extracting Protein: The seed or part of plant from which the protein is to be extracted

must be finely ground before the process of extraction is effected to insure securing maximum yield and to effect complete extraction in the shortest possible time. In case the ground plant substance is oily it must be extracted several times with ether until entirely free from oil. The method employed intthe present investigation is a modification of the Soxhlet method.

Solvents and Methods Used for Extraction of Plant Proteins: The solvents usually employed for the extraction of proteins from plants are water, neutral saline solutions, 70% to 80% alcohol and very dilute acids and alkalies.

Some common proteins extracted from plants by means of water are albumins and proteoses. When this aqueous extract is saturated with $(NH_4)_2SO_4$ the proteins are precipitated.

In the case of the extraction of plant substance with solutions of neutral salts a 10% NaCl solution is a solvent frequently employed. Osborne (11) states that "the general plan followed in conducting an extraction with sodium chloride solution is to treat the ground seed with a sufficient proportion of the solvent so that it will subsequently yield an extract of which so much can be filtered clear as to be equal to about three-fourths of the volume of the solvent applied to the meal. The proportion necessary to attain this end depends not only on the amount of the insoluble residue but also on the proportion of water with which it combines. It also depends to a large extent on the character of the solution which the soluble constituents

of the seed yield, for some varieties of seeds contain substances which produce extremely gummy, viscid solutions which render filtration extremely difficult. No general statement can be made as to the proper proportion of solvent, or the methods to be employed in filtering the extracts, as each kind of seed requires special treatment. A filtered extract may be subjected at once to dialysis and the dissolved globulin thus separated. The extraction may also be made with warm dilute saline solutions and the globulin precipitated by cooling the filtered extract as, in most cases, the globulins are much less soluble in cold solutions than in warm. This method of treatment often results in the production of crystalline preparations which can be afterwards recrystallized from warm dilute saline solutions and brought to a high state of purity". Other salts than NaCl which may be employed for extracting proteins are: NH_4Cl, $BaCl_2$, $CaCl_2$, KBr, KI, $MgSo_4$, ammonium oxalate, potassium ferro cyanide and organic salts---ammonium benzoate.

The amount of protein extracted by alkalies and acids is usually greater than that extracted by water or neutral salts. This is due to the fact that alkalies and acids dissolve proteins enclosed in places inaccessible to salt solutions. This method is disadvantageous because alterations in the proteins may result by the action of acids or alkalies if their concentrations exceed certain limits.

Edestin a typical globulin: Edestin conforms strictly to the

definition of globulin and it is one of the most typical and abundant of vegetable globulins. The reason for selecting edestin for the study of the phenomenon of heat coagulation have been already mentioned. It was first prepared by Barbiesi from squash seed and then it was prepared in a crystallized form by Grubler. Ritthausen (13) extracted it from hemp seed, castor bean and sunflower. T. B. Osborne (11) extracted odestin from the seeds of flax, cotton, wheat, rye, barley and maise.

Edestin is soluble in 10% sodium chloride solution. Its solubility, however, is much more pronounced in warm brine solution than in a cold one. The solvent effect of different quantities of salts on edestin were determined by Osborne (11) and I. F. Harris as fllows: "Portions of two grammes each of pure crystallized ed estin were suspended in sufficient water to make a final volume of 20 c. c. with the different quantities of molar solutions of the several salts which were afterwards added, the edestin being in each case in excess of the amount dissolved. After agitating for some time, the solutions were filtered, nitrogen was determined in 10 c. c. of each, and the amount of edestin dissolved was calculated from the nitrogen in solution. It was thus found that the amount of dissolved edestin was closely proportional to the concentration of the salt solution. Its solubility in solutions of sodium, potassium and calcium chlorides was nearly the same. In solutions of magnesium, calcium, strontium, or barium chloride its solubility was twice as great as it was in solutions of the chlorides of the monovalent

bases, with the exception of the lithium chloride, in solutions of which it did not dissolve as abundantly as in those of the chlorides of the other monovalent bases. The sulphates of potassium, sodium, lithium and magnesium had a solvent power corresponding closely with that of the chlorides of the bivalent bases. Bromides and iodides did not behave like chlorides, for sodium and potassium iodides had a solvent power twice as great as that of the corresponding chlorides, agreeing in this respect with the chlorides of the bivalent bases. The bromides were less energetic solvents than the iodides but more energetic than the chlorides. Barium and calcium bromides were equal to one another in solvent power but this was less than that of sodium or potassium iodide and greater than that of sodium or potassium bromide, the two latter being somewhat less powerful solvents than the corresponding chlorides".

Solubility of edestin in acid and bases: Edestin is a compound which is amphoteric in character and which for this reason combines with both acids and bases.

Pure edestin when free from combined acids or bases is insoluble in water. If this edestin is suspended in pure water and hydrochloric acid is added in the proportion of two mols. of hydrochloric acid to one mol of edestin, it was found by Osborne (11) that no solution of edestin takes place until one-half of the amount of the acid has been added. On adding the second half of the acid, solution takes place at a rate proportional to

the amount of acid added. The acidity of the solution obtained with the second half of the acid increases at twice the rate at which the acid is added. When all the second half of the acid is added edestin dissolves completely. If one ceases to add acid before all edestin dissolves and if one examines the solution and the insoluble parts one will find that the acidity of the part insoluble in water is equal to that of a compound of one molecule of edestin to one molecule of hydrochloric acid, while the acidity of the part soluble in water is equal to a compound of two molecules of hydrochloric acid to one molecule of edestin. Edestin, therefore, forms salts corresponding to edestin monochloride and edestin bichloride. Edestin also functions as an acid. Thus it dissolves in alkali in molecular proportions forming alkaline salts. When edestin is extracted from plants by neutral salts the product thus obtained was shown by Osborne (11) to consist not of the free edestin but of a mixture of its salts. Thus edestin contains chiefly edestin chlorides when crystallized from sodium chloride solution and sulfates when crystallized from solutions containing ammonium sulfate. That edestin contains chiefly edestin chlorides when extracted by NaCl solution is shown by the fact that when the thus extracted protein is treated with pure water a part of it goes into solution. The part that goes into solution is twice as acid as the insoluble part. The soluble protein is edestin bichloride.

Properties of edestin monohydro-chloride: According to Osborne

(11) edestin monohydro-chloride is wholly insoluble in water but dissolves in 10% sodium chloride solution and it is readily thrown out from solution by adding water. On warming this solution the precipitate dissolves and appears on cooling. Edestin monohydro-chloride conforms to the definition of globulin.

Properties of edestin bihydro-chloride: According to Osborne (11) edestin bihydro-chloride is soluble in water and it is precipitated from this solution by addition of small quantities of alkali salts. It is insoluble in one or two per cent sodium chloride solution.

Precipitation of edestin from edestin monohydro-chloride and edestin in bihydro-chloride: When edestin monohydro-chloride or edestin bihydro-chloride is suspended or dissolved in water and this made neutral to phenolphthalein edestin separates out completely. When this solution is filtered and filtrate evaporated to dryness and analyzed it will be found to consist of the chloride salt of the alkali used in neutralization. If the edestin was originally crystallized from ammonium sulfate rather than sodium chloride the product will be potassium sulfate.

Precipitation of edestin from 10% sodium chloride solution: Edestin dissolved in 10% sodium chloride is precipitated completely when saturated with sodium sulfate at 34°. It is partially precipitated when saturated with sodium chloride and is precipitated more completely when saturated with magnesium sulfate.

Composition, formula, and molecular weight of edestin: When edestin is dried at 110° and analyzed, its composition is found

by Osborne (11) to be as follows:

Carbon	51.55%
Hydrogen	6.92%
Nitrogen	18.67%
Sulphur	0.90%
Oxygen	21.96%

Edestin has a molecular weight of 7,138, twice which is 14,276. The formula being carbon 614, hydrogen 988, nitrogen 190, sulphur 4, oxygen 196.

EXPERIMENTAL

Extraction of edestin from hemp seed: The method followed for the extraction of edestin from hemp seed is that outlined by Morrow (10) with minor modeifications which were found desirable to apply in order to get edestin of high purity.

750 c. c. of 10% sodium chloride solution were heated to 65° C. and, while stirring continuously, 250 grams of the ground ether extracted and air dried hemp seed were added in small amounts at a time and then allowed to stand in a water bath at 70° for an hour. The substance was stirred frequently during this time. A piece of clean cloth was then moistened with hot 10% sodium chloride solution and a portion of the mixture was then placed in it and squeezed by the hand until all the liquid was wrung out in a clean beaker placed in a water bath at 70°. This process was repeated with new portions of the mixture until

all the solution was squeezed out of all the mixture. The solution or extract thus obtained, when heated to a temperature of 65° C., was filtered through a Buchner funnel which was previously prepared as follows: A Buchner funnel which was fitted to a suction flask which, in turn, was connected to a suction pump, was fitted with filter paper. A hot 10% sodium chloride solution was poured on the filter paper while the suction pump was in operation, thus causing it to adhere closely to the funnel. Decolorizing carbon was then placed on the filter paper and hot 10% sodium chloride solution was poured into the funnel until a completely moistened mat of decolorizing carbon was formed. The Buchner funnel was kept warm by a simple but effective device. This device consisted of winding as many times as possible a rubber tubing connected at one end to a steam source. When steam was allowed to pass through the rubber tube the temperature of the content of the funnel was easily kept at 60° C. The reason for using the decolorizing carbon was to decolorize the extract which was slightly colored when obtained as mentioned above. Some edestin was lost by being adsorbed by the decolorizing carbon, but this was sacrificed for a purer product. The extract was filtered through the above mentioned Buchner funnel and the clear filtrate obtained was cooled to room temperature and then placed in a refrigerator for a day or two. When the edestin had crystallized out it was filtered through another Buchner funnel. The edestin crystals were then washed several

times with cold ten per cent sodium chloride solution and then was returned to a clean flask, dissolved in ten per cent sodium chloride at 65° C., filtered, cooled to room temperature and placed in the refrigerator as before. This process was repeated several times and it yielded edestin of high purity. The edestin was then washed with 50% ethyl alcohol, 95% ethyl alcohol and absolute ethyl alcohol and finally with ether. The edestin was then dried and bottled for use.

PART IV.

Heat Coagulation of Edestin

In asmuch as compounds which are known to contain amide groups are decomposed by water when such compounds are heated in certain aqueous media, it was thought advisable to investigate the possibility of amide groups of proteins being decomposed by water and thus give rise to ammonia during heat coagulation. Egg albumin can not be used for such a study due to the fact that ammonium salts are used in its extraction. Indeed when dialyzed egg albumin solution is heated it gives rise to some ammonia. The source of such ammonia is, most probably, ammonium sulfate which is used in the extraction of albumin. It is necessary, therefore, to choose a protein in the extraction of which and in the purification of which no ammonium compound is used.

Preparation of Edestin

Edestin was prepared in the same manner as outlined before and precautions were taken so that the materials used for its extraction and purification were free from ammonia. Thus edestin was prepared in an atmosphere which was tested and found to be free from ammonia. The sodium chloride used was of high purity and ammonia free. The water employed was conductivity water which was shown to be free from ammonia.

Apparatus Designed for the Detection of Ammonia and Carbon Dioxide

The apparatus devised for the estimation of ammonia eliminated during the heat coagulation of edestin consisted esentially of vessels in which air was scrubbed for its carbon dioxide and ammonia content, of vessels in which suitable tests for these constituents could be made, of a vessel in which the heat coagulation could be carried out, and of a vessel in which the ammonia, if it were to be given off, could be caught. The system was suitably connected by means of glass tubes, which were connected with rubber tubing, to permit the entire system to be swept with a stream of ammonia and carbon dioxide free air. Figure I is a photographic picture of the apparatus used in this study.

The important parts of the apparatus are lettered in the figure and the names assigned to them are given in the following legend:

(A) Carbon dioxide (air) absorber

(B) Ammonia (air) absorber

(C) Carbon dioxide test tube

(D) Ammonia test tube

(E) Lime water funnel

(F) Dilute sulfuric acid funnel

(G) Coagulating chamber

(H) Foam collector

(I) Ammonia absorber

(J) Concentrated sulfuric acid driers


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