THE EFFECT OF SUGARS ON SOME PHYSICAL AND CHEMICAL PROPERTIES OF EGG ALBUMIN

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ABSTRACT

It previously was shown that several simple sugars, one corresponding alcohol and a disaccharide inhibit the precipitation of egg albumin brought about by heat at 70°C. and pH 4.8. Since no more protein precipitated when the sugar was dialyzed out of the mixture these experiments were interpreted as meaning that the denaturation of egg albumin was inhibited rather than the precipitation of otherwise denatured material.

Native egg albumin does not react with typical sulfhydryl reagents whereas denatured egg albumin reacts with such reagents in a quantitative manner. It previously was shown that sugars and related compounds also inhibit the liberation of sulfhydryl groups in egg albumin brought about by heat. Since sugars do not interfere in the reaction of free sulfhydryl groups with such reagents these experiments were taken as further proof that sugars and related compounds protect egg albumin against the denaturing effects of heat.

The measurements of sulfhydryl groups in the experiments above indicated a cysteine content of 0.59 per cent in denatured and precipitated egg albumin. Later methods of measuring sulfhydryl groups have indicated a cysteine content of 1.2 per cent in denatured egg albumin which was kept in solution during the measurement. Adapting these latter methods the present work has shown that 1.0 M glucose, galactose, fructose or sucrose prevent about 30 per cent of the

sulfhydryl groups usually found in egg albumin heated at 50°C. and pH 1.0 from appearing. This is further proof that sugars protect egg albumin against acid-heat denaturation.

When guanidine hydrochloride or sodium dodecyl sulfate is used to denature and keep egg albumin in solution for sulfhydryl measurement, the sugars glucose, fructose, galactose or sucrose do not affect the amount of sulfhydryl groups usually measured. This is evidence that sugars do not affect the denaturation of egg albumin brought about by either of these reagents.

Since charged groups in the protein molecule are usually thought to be important factors in denaturation, it seemed possible that the sugars covered up some critical charged groups and thus affected egg albumin denaturation. Such a circumstance could be indicated electrophoretically.

Electrophoresis experiments did not demonstrate any combination between egg albumin and glucose, fructose, sucrose, or mannitol detectable as a new component. This indicates that the sugars do not in any way affect the charged groups in egg albumin in preventing denaturation.

Egg albumin heated at 50°C. and pH 3.0 showed two components when analyzed electrophoretically at pH 3.0 in glycine-hydrochloric acid buffer. One of these components was precipitated at pH 4.8 and thus was indicated to be denatured egg albumin while the other component was soluble at pH 4.8 and thus was indicated to be native egg albumin. The precipitable component was shown to form slowly when egg albumin was heated in the presence of 1.0 M glucose, fructose mannitol or glycine. Approximately the same amount of denaturation occurred in twenty-four hours in the presence of glucose or mannitol as occurred in twenty minutes in egg albumin alone when heated at 50°C. and pH 3.0. Electrophoresis was thus shown to be another means of indicating that sugars and related compounds protect egg albumin against denaturation by heat.

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INTRODUCTION

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Neuberg (1) in 1916 described the characteristic ability of water solutions of numerous salts to dissolve otherwise insoluble materials. He called this property hydrotropic and pointed out that a wide range of materials including the proteins were affected by it. He described in particular several experiments with sheep blood serum and egg yolk in which some hydrotropic salts, such as sodium benzoate and sodium salicylate, maintained a clear solution even when the mixture was heated.

Later, following the lead of colleagues who had shown that several anticoagulants protected the biochemical properties of diptheria toxin and antitoxin against heat, Beilinsson (2) proposed to determine whether all the physical-chemical properties of typical proteins were preserved by the presence of such hydrotropic materials. He worked with two protein preparations, 1) rabbit serum and 2) five per cent egg albumin in physiological saline, and two anticoagulants, 1) glycerol and 2) sucrose. The method of analysis was titration of aliquot samples with saturated ammonium sulfate solution to a standard turbidity. By plotting the milliliters of saturated ammonium sulfate required against time of heating, Beilinsson was able to show the following: 1) The stability of the protein materials decreased as the temperature increased. 2) The stability increased as the concentration of the stabilizing agent increased. 3) Sucrose was a considerably better stabilizing agent than glycerol. In fact, rabbit serum saturated with sucrose was almost completely stable for one hour at 62°C. and pH 7.1, and egg albumin saturated with sucrose was stable for one hour at 75°C. and pH 7.1. Experiments, in which the quantity of nitrogen in the protein precipitated at the isoelectric point (pH 5.3) was determined, confirmed the results given above.

Duddles (3) in this laboratory expanded the work of Beilinsson by using several hexose sugars and one corresponding alcohol as well as sucrose. Denaturation and subsequent coagulation of the protein, egg albumin, was accomplished by heating at 70°C. for ten minutes at pH 4.8. The quantity of nitrogen in an aliquot of the filtrate was then determined. The results showed the following: 1) In all cases the amount of nitrogen in the filtrate increases as the concentration of the stabilizing agent increased. 2) Glucose was the best stabilizing agent followed closely by fructose; egg albumin saturated with either glucose or fructose was almost completely stable to heat at 70°C. for ten minutes at pH 4.8; mannitol was fairly effective and mannose was slightly effective. 3) Glucose stabilized egg albumin to coagulation caused by ultra-violet light at pH 4.8. 4) Coagulated egg albumin was not repeptized by saturation with glucose and subsequent standing for twenty-four hours.

The work of Beilinsson and Duddles merely proved that sugars and sugar alcohols inhibited the heat coagulation of egg albumin; proof that denaturation was prevented was lacking. Hardt (4) approached the problem by using the fact, first demonstrated by Arnold (5), that heat denatured egg albumin does react with sulfhydryl reagents whereas native egg albumin does not. Two methods were used to measure sulfhydryl groups: 1) The method of Rosner (6) using iodoacetate and 2) The method of Todrick and Walker (7) using phenolindo-2-6-dichlorophenol. The first method seemed more reliable but the second method confirmed the results of the first. Denaturation was caused by heat at 70°C. for fifteen minutes at pH 4.8. The following observations were made: 1) The number of sulfhydryl groups liberated decreased as the concentration of sugar, ranging from 0.045 M to 0.450 M, increased. 2) Glucose was the best stabilizing agent with fructose and mannose following; mannitol was not nearly so effective; arabinose was as effective as fructose or mannose but xylose was slightly less effective. 3) Both arabinose and xylose stabilized egg albumin when a method of analysis similar to the one employed by Duddles was used, but xylose was less effective. 4) Egg albumin was more stable at pH 8.6 than at pH 4.8. 5) The effectiveness of glucose or fructose in stabilizing egg albumin was not improved by exposing the protein to contact with the sugar for periods up to ninety-six hours before denaturation by heat. 6) No protein-carbohydrate combination could be demonstrated.

The work of Beilinsson, Duddles, and Hardt had demonstrated by three very different methods of analysis that several sugars inhibit to a greater or lesser degree the denaturation and subsequent coagulation of egg albumin. Yet the nature of this inhibition had not been explained. Hardt had found only 0.590 per cent cysteine in denatured coagulated egg albumin using the iodoacetate method. This value agreed quite well, however, with the results of several other workers (4) including those by Mirsky and Anson (8) who gave values of 0.56 per cent and 0.616 per cent cysteine for egg albumin with two different respective methods. Later, however, Anson (9) showed that 1.2 per cent cysteine could be measured in denatured egg albumin dispersed by the denaturing agent. He achieved this result with very different titrating agents, each in the presence of several different denaturing agents, all of which kept the denatured protein in solution. "The fact that very varied procedures yield the same titration value is strong evidence that only sulfhydryl groups are being titrated." (10). Since twice as many sulfhydryl groups were revealed by these methods as by the methods used by Hardt with coagulated egg albumin, Anson (11) had suggested that the inhibiting action of sugars should be determined by some of the latter methods to make sure of the validity of the previous work. It was with this suggestion in mind that the first portion of the experimental work was undertaken.

Luck and co-workers (12, 13, 15) performed a considerable amount of work with the heat stabilization of human serum albumin and bovine serum albumin during World War II. They worked mostly with 25 gram per cent protein solutions because that was the form in which the human serum albumin was manufactured and distributed for use by the Armed Forces. A rapid micro-method of analysis was developed in which the solutions were heated in thin-walled capillary tubes and the "cloud point" time observed (12). They found that the basic, requisite structure for stabilizing properties was an anion with a non-polar group. The maximum stabilization was attained with salts of fatty acids 7 to 8 carbon atoms in chain length and in a concentration of about 0.15 M. Cations with the same non-polar groups increased the susceptibility of serum albumin to heat denaturation. It was shown that these effects were the same on both sides of the isoelectric point of albumin. The strength of the acid group did not markedly affect the amount of stabilization since the relative effect of the sulfonic and carboxy acids with alkyl groups of the same chain length was about the same. Viscometric studies showed that the fatty acids prevented viscosity increases in heated solutions of albumin thus demonstrating that the fatty acids stabilized native and not denatured protein. Ultrafiltration studies permitted the determination of the amount of bound fatty acid anion in a solution containing both serum albumin and the sodium salts of lower fatty acids. It was found that the amount of combination of fatty acid anions with serum albumin increased markedly with an increase in the chain length of the fatty acid. The amount of combination of caprylate was decreased considerably if the albumin was denatured by urea prior to the addition of the caprylate. Electrophoretic studies (16) demonstrated a specific interaction of the fatty acid anions with albumin with an increasing affinity in ascending from butyrate to caprylate.

Lundgren *et al.* (17) had demonstrated the formation of complexes between egg albumin and detergents of the alkylaryl-sulfonate type in solutions alkaline to the isoelectric region. These complexes exhibited well-defined electrophoretic boundaries whose mobilities ranged between those of the protein and detergent. Putnam and Neurath (18) were able to show two complexes between horse serum albumin and purified sodium dodecyl sulfate which had well-defined electrophoretic boundaries. The formation of these complexes was dependent upon the weight ratio of protein to detergent rather than on their absolute concentrations. This finding had been confirmed by precipitation and viscosity studies on horse serum albumin and sodium dodecyl sulfate mixtures. Although no complexes of protein and non-ionic materials had been demonstrated previously it was this work of Putnam and Neurath which prompted the second portion of the present work, that is, an attempt to demonstrate complex formation between egg albumin and various sugars by means of the electrophoresis apparatus.

EXPERIMENTAL - PART ONE

EXPERIMENTAL - PART ONE

Native egg albumin neither gives a detectable nitroprusside color for sulfhydryl groups nor will it react with such mild oxidizing agents as porphyrindin, ferricyanide ion, and tetrathionate ion. When egg albumin is denatured by heat or by a number of other physical and chemical agents, it readily gives a nitroprusside color test for sulfhydryl groups and will react with several reagents in a quantitative manner. Consequently the measurement of sulfhydryl groups can be used to determine the amount of denaturation which has taken place in a given amount of egg albumin. As mentioned previously, Anson (19) has developed several methods of measuring sulfhydryl groups which indicate twice the number of sulfhydryl groups that previous methods have shown. It was the purpose of the first part of the experimental work reported in this dissertation to adapt the methods of Anson so that the effect of sugars on the amount of denaturation of egg albumin could be observed better.

MATERIALS

All inorganic chemicals met either C.P. or A.C.S. specifications and were products of either the J.T. Baker Chemical Company or Baker and Adamson. The sugars used were C.P. products of the Pfanstiehl Chemical Company. The simple sugars were the natural isomers.

Several samples of egg albumin were prepared by the method of Kekwick and Cannon (20). All were recrystallized three times and then redissolved in a minimum amount of water. Part of some preparations was kept for extended periods of time under toluene in the refrigerator. All solutions were dialyzed against distilled water to remove sodium sulfate (which was occluded with the crystalline egg albumin) before the solution was used in experiments. Dialysis was done both at room temperature and in the refrigerator but in all cases was continued until the specific conductance of the albumin solution was less than 2 x 10^{-4} mho. After dialysis, solutions were kept under toluene in the refrigerator. The final concentration was determined by nitrogen analysis (21) or (22).

The methods developed by Anson (19) utilizing potassium ferricyanide were the simplest to perform; the reagent was readily obtainable and stable in solution. Other procedures developed by Anson with p-chloro-mercuribenzoate and tetrathionate ions were used by him only to confirm the results which he obtained with potassium ferricyanide. In several procedures using the latter reagent the results were obtained by developing Prussian blue from the ferrocyanide previously formed when ferricyanide reacted with the sulfhydryl groups of denatured egg albumin. The amount of Prussian blue was determined by taking a reading in a suitably calibrated photelometer.* It was necessary, therefore, to have a solution of potassium ferrocyanide which could be used as a reference standard.

* A photoelectric colorimeter.

Three procedures were used for making potassium ferrocyanide stock solutions during the course of this work. 1) The potassium ferrocyanide contained three moles of water of crystallization and had a molecular weight of 422.33. Thus, 1.0558 g. made to 250 ml. resulted in a 0.01 M solution. 2) The same lot of potassium ferrocyanide was heated to constant weight at 105-110°C. It was then assumed to have no water of crystallization and a molecular weight of 368.272. When 1.8412 g. was made to 100 ml. a 0.05 M solution resulted. Sodium carbonate in a concentration of 0.2 per cent was added to this solution as a stabilizer. 3) For the greater part of this work the amount of potassium ferrocyanide in accurately weighed samples was determined by titration with standard potassium permanganate solution (23). From this titration the material was calculated to contain 101.6 per cent $K_4Fe(CN)_6 \cdot 3\ H_2O$, indicating a partial dehydration. Therefore subsequent stock potassium ferrocyanide solutions were made of 1.0394 g. of this salt diluted to 250 ml. to give a 0.01 M solution. These solutions were stabilized by 0.2 per cent sodium carbonate. Fresh potassium ferrocyanide stock solutions were usually made monthly; all solutions were diluted to 0.001 M for use as reference standards.

Potassium ferricyanide solution was made according to the directions of Anson (19). The stock solution was 0.4 M and any ferrocyanide present was oxidized by treatment with bromine. The ferricyanide solution was stored in the refrigerator and usually

1.25 ml. of the solution was diluted to 10 ml. to make a 0.05 M solution for use. The stock solution was tested occasionally for ferrocyanide with ferric sulfate and subsequent development of Prussian blue.

Ferric sulfate solution, used to develop Prussian blue from ferrocyanide, was made according to Folin and Malmros (25).

Sulfuric acid in a concentration of 1.0 M was made and standardized. It was used to stop the oxidising action of potassium ferricyanide when the amount of sulfhydryl groups was to be determined by the development of Prussian blue.

Two buffers were used during the course of this work: 1) a neutral buffer, pH 6.7-6.8, consisting of equal molar parts of 1 M NaH_2PO_4 and 1 M Na_2HPO_4, and 2) a buffer, pH 6.3, consisting of one part 1 M Na_2HPO_4 and three parts 1 M NaH_2PO_4. The pH of each of these buffers was measured on the Beckman pH meter.

DETECTION OF DENATURATION

In the procedures developed by Anson (19) involving the development of Prussian blue the usual reagents were as follows: 1) 0.5 ml. of two per cent egg albumin or 1.0 ml. of one per cent egg albumin; 2) neutral phosphate buffer varying in amount from two drops to 0.2 ml. depending on the procedure; 3) ferricyanide solution which varied from one drop of 0.5 M solution to 0.5 ml. of 0.1 M solution;

4) and the denaturing agent which could be urea or sodium dodecyl sulfate. These reagents were allowed to stand together at 37°C. for a suitable length of time varying from one minute for sodium dodecyl sulfate to five minutes for urea. This period of time was to permit the reaction between denatured egg albumin and ferricyanide to occur at a neutral pH. The reaction was stopped by the addition of 0.5 ml. of 2 N sulfuric acid, then water was added to a volume of 9.5 ml., and finally 0.5 ml. of ferric sulfate was added to develop Prussian blue. After twenty minutes the amount of Prussian blue was determined by use of a "photelometer" using light transmitted by a red filter. The Hellige-Diller "photelometer" set at 660 mμ was the instrument used in the present work for measuring the concentration of Prussian blue.

As indicated above the usual amount of egg albumin was 10 mg. which, upon denaturation, reacted with ferricyanide to form one ml. of 0.001 M ferrocyanide. Therefore, amounts of ferrocyanide from one ml. to 0.0 ml. were used with a final volume of reagents to ten ml. to prepare a standard curve. The procedure was as follows: To measured quantities of 0.001 M potassium ferrocyanide were added 0.2 ml. of buffer, pH 6.8 or pH 6.3; 0.5 ml. of ten per cent sodium dodecyl sulfate; from one drop to 1.0 ml. of 0.05 M ferricyanide; water to nine ml. and finally 0.5 ml. of 2 N sulfuric acid and 0.5 ml. of ferric sulfate. After twenty minutes the per cent transmission was measured in the Hellige-Diller "photelometer" at 660 mμ.

Such curves were run several times during the course of the work always with the same results. The presence of egg albumin did not change the values when the experiment was arranged so that the denatured material and ferricyanide could not react.

No attempt was made to keep the ionic strength constant in the experiments reported in this part of the dissertation. Several reagents, i.e. buffer, detergent, and potassium ferricyanide, which had an effect on the ionic strength were present; the variability in the amounts of these reagents present from experiment to experiment made the maintenance of a constant ionic strength inexpedient. Furthermore, Luck and coworkers (13) had found that ionic strength was not an important factor in their work with heat denaturation of serum albumin. Such an assumption may be questionable.

DENATURING AGENTS

1. Urea.

Urea which was the denaturing agent in some preliminary experiments was used as obtained.

A few experiments were carried out using urea as the denaturing agent but these experiments were discontinued when it was discovered that egg albumin precipitated at the time of the Prussian blue development. Further, the large quantities of urea needed to bring about denaturation made it an inconvenient experimental procedure.

2. Guanidine Hydrochloride.

Experiments with guanidine hydrochloride used ferricyanide as a titrating agent and the nitroprusside test to indicate the end-point. The typical procedure was as follows: To 0.5 ml. of two per cent egg albumin there were added 0.1 ml. of buffer (pH 6.8), measured quantities of ferricyanide, and 1.2 g. of guanidine hydrochloride. After three minutes in a 37°C. bath the solution was cooled in ice water and then one drop of five per cent nitroprusside and one drop of twenty-seven per cent ammonia added. The first tube in which no pink color developed indicated the amount of ferricyanide necessary to abolish all SH groups.

Guanidine hydrochloride was made from guanidine carbonate in the manner described by Anson (19). Several samples were prepared, each time from 100 g. of guanidine carbonate. Except for the first preparation in which a metal propellor stirrer was used, all samples were prepared with glass stirrers. With the exception of the first sample these preparations of guanidine hydrochloride conformed to two criteria stated by Anson (19). 1) "The color given by egg albumin and nitroprusside in guanidine hydrochloride solution——should not be increased if one drop of 0.1 N cyanide is present. 2) The same amount of ferricyanide should be required to abolish the nitroprusside test of denatured egg albumin in guanidine hydrochloride solution whether the ferricyanide is added before or thirty minutes after the addition of guanidine hydrochloride." Anson (10) had suggested that when these criteria are not met it is probably due to metallic impurities in the guanidine hydrochloride which promote oxidation of protein sulfhydryl groups by oxygen in the air.

When guanidine hydrochloride was used as a denaturing agent, potassium ferricyanide solution was used as a titrating agent for sulfhydryl groups with nitroprusside as the indicator of the end-point. Accordingly, the concentration of the potassium ferricyanide had to be accurately known, the concentration of the stock solution being determined by a method given in Treadwell and Hall (24). For actual use in these titrations 0.25 ml. of 0.40 M potassium ferricyanide solution was diluted to 50 ml. to make a 0.002 M solution.

3. Sodium Lauryl Sulfate.

A detergent, sodium lauryl sulfate, prepared by the Amend Drug and Chemical Company, 117-119 East 24th St., New York, 10, N.Y. was used as the denaturing agent in many experiments. This was made up at room temperature as a ten per cent stock solution. In referring to it hereafter the more exact term sodium dodecyl sulfate will be used. Anson (19) had used Duponol PC which, although consisting mainly of the C_{12} compound, sodium dodecyl sulfate, was a mixture of the C_{10}-C_{18} compounds. It was felt that the more homogeneous product would be more satisfactory than Duponol PC.

4. Heat.

In addition to the methods utilizing urea, guanidine hydrochloride and sodium dodecyl sulfate as denaturing agents which keep egg albumin in solution, Anson (28) developed a heat and acid denaturing procedure (which did not coagulate egg albumin) that revealed as many SH groups as the chemical denaturants. In this procedure 1 ml. of 1.0 N hydrochloric acid was added to six ml. of two per cent egg albumin which was then placed in a 50°C. bath for five minutes. The mixture was cooled in ice water, a slight excess of 1.0 N sodium hydroxide added, and finally diluted to one per cent concentration of egg albumin. The sulfhydryl groups were detected by adding ferricyanide to buffered one ml. aliquots of the diluted mixture and after five minutes at 37°C., developing Prussian blue in the usual manner.

RESULTS - PART ONE

1. Denaturation by Guanidine Hydrochloride.

To determine the effect of sugars, egg albumin solution was made 2 M with respect to either glucose or fructose and guanidine hydrochloride was used as the denaturing agent. The procedure was then the same as described previously. Neither of these sugars had any effect on the quantity of ferricyanide needed to abolish the sulfhydryl groups revealed by guanidine hydrochloride. The titration was the same whether egg albumin alone or egg albumin which had been made 2 M with glucose or fructose was used. Storage of the egg albumin and sugar mixture overnight in the refrigerator did not change the titration value. Titration of an amount of cysteine hydrochloride equivalent to one ml. of one per cent egg albumin gave the same value as for egg albumin alone.

Since previous workers had indicated that fructose and glucose were the most effective sugars inhibiting the denaturation of egg albumin by heat, the experiments with guanidine hydrochloride were not continued with other sugars.

2. Denaturation by Sodium Dodecyl Sulfate.

The experiments in which sodium dodecyl sulfate was the denaturing agent used the production of Prussian blue as the means of analysis. In the earliest experiments a direct comparison between egg albumin in the absence of sugar was made with egg albumin in the presence of sugar. The total volume of reagents at the time of the reaction with ferricyanide was 4.0 ml. Experiments were carried out with 0.2 M, 0.5 M and 1.0 M glucose and 0.5 M and 1.0 M fructose at the time of the ferricyanide reaction. Fifty mg. of sodium dodecyl sulfate and 0.01 mM of ferricyanide were added. The reaction was allowed to proceed for about one and one-half minutes before 1.0 M sulfuric acid was added. A blank containing egg albumin and sugar but no sodium dodecyl sulfate at the time of the ferricyanide reaction was used. The values obtained for egg albumin and sugar were the same as the values obtained for egg albumin alone. In every case, however, in the presence of sodium dodecyl sulfate alone only about eighty to eighty-five per cent of the sulfhydryl groups that should have been present were revealed. This amount was not increased when the time of reaction with ferricyanide was increased to five minutes. Although these results are low according to Anson's standards, Mirsky (26) reported this quantity of sulfhydryl groups utilizing essentially the same methods used by Anson. Anson (27) in commenting on this report said that he has "Found an occasional sample of recrystallized egg albumin which had a low SH content."

The fifty mg. of sodium dodecyl sulfate, which was used in the experiments above, was not only enough to denature egg albumin but also to keep it in solution when acid ferric sulfate was added at the end to develop Prussian blue. The question arose as to whether smaller amounts of sodium dodecyl sulfate, which would still completely denature egg albumin alone, might permit the protective effect of sugar to be exhibited. Experiments to determine the least amount of sodium dodecyl sulfate which would completely denature egg albumin showed that twelve mg. was the smallest quantity that could be used in a total volume of 1.7 ml. at the time of the ferricyanide reaction. Additional detergent was added after the reaction had been stopped with acid to keep the egg albumin in solution. Ten minutes reaction time with 0.025 mM of ferricyanide was allowed. The concentration of sugar at the time of the ferricyanide reaction with SH groups was approximately 0.6 M. Neither glucose nor fructose affected the amount of denatured egg albumin.

It became apparent in these experiments that the sugars as well as egg albumin were reacting with ferricyanide. Some experiments were then carried out in which tubes containing 1.0 ml. of ferrocyanide (the equivalent of 1 ml. of 1 per cent egg albumin) and sugar could be compared with tubes containing egg albumin and sugar. Ten minutes reaction with 0.025 mM of ferricyanide in a total volume of 5.0 ml. and a concentration of glucose and fructose of 1 M was allowed. No protective effect was demonstrated whether the amount of sodium dodecyl sulfate present was 50 mg. or 12 mg.

The reaction of ferricyanide in alkaline medium with reducing sugars is the basis for their quantitative measurement. This reaction apparently still proceeds to a very small extent at pH 6.8. In an effort to reduce this reaction without affecting the reaction between ferricyanide and sulfhydryl groups, a buffer pH 6.3 and only 0.002 mM of ferricyanide were used in a total volume of 5.0 ml. A reaction time of two minutes proved to be sufficient for measurement of all SH groups in egg albumin. Experiments were then carried out in which tubes containing all reagents except egg albumin but with sugar could be used as blanks for tubes containing egg albumin with sugar. Blanks containing all reagents with egg albumin and sugar but no detergent until a ferricyanide reaction had been stopped were also used. The sugar concentration at the time of the ferricyanide reaction was 1 M. Again, neither glucose nor fructose nor sucrose, had any effect in protecting egg albumin against denaturation by sodium dodecyl sulfate as measured by the amount of sulfhydryl groups revealed.

The concentration of egg albumin in the experiments immediately above was only 0.2 per cent at the time of reaction with ferricyanide. Experiments were next carried out in which the concentration of egg albumin was 1.0 per cent, sugar was 1 M with 10 mg. of sodium dodecyl sulfate and 0.002 mM of ferricyanide in a total volume of 1.0 ml. A reaction time of five minutes was used. Practically no reaction between ferricyanide and reducing sugar occurred under these circumstances. Experiments with glucose, galactose, and sucrose showed that none of these sugars in a concentration of 1 M inhibited the denaturation of egg albumin.

3. Denaturation by Heat and Acid.

The first acid-heat denaturation experiments were carried out in an open test tube with occasional shaking. The full amount of sulfhydryl groups was not detectable, however, and it was found that all the sulfhydryl groups could be detected regularly if the denaturation was carried out in a Thunberg tube in which the atmosphere was replaced with nitrogen. This procedure was carried out with egg albumin made 1 M with glucose. This made the concentration of sugar about 0.86 M at the time of denaturation. Under these circumstances only about seventy-nine per cent of the sulfhydryl groups were revealed. When the procedure was carried out with a 1.0 M glucose concentration at the time of denaturation, sixty-nine per cent of the sulfhydryl groups were revealed. In order to be sure that this reduction in the amount of sulfhydryl groups was not due to oxidation taking place during the denaturing procedure one ml. samples of acid-heat denatured egg albumin were allowed to react with ferricyanide in the presence of 0.5 ml. of ten per cent sodium dodecyl sulfate. The amount of sulfhydryl was then the same as for one ml. of one per cent egg albumin denatured only with sodium dodecyl sulfate.

This modified acid-heat denaturation was carried out in the presence of 1 M galactose, 1 M fructose, and 1 M sucrose, the concentrations present when the acid-heat denaturation was accomplished. The average amount of sulfhydryl groups revealed in the presence of each of these sugars is as follows: 1 M galactose 70 per cent, 1 M fructose 74 per cent, 1 M sucrose 72 per cent.

DISCUSSION -PART ONE

DISCUSSION - PART ONE

In all experiments ferricyanide reacted with sulfhydryl groups to form ferrocyanide. In some experiments the ferricyanide ion reacted in a small degree with the reducing sugar present forming more ferrocyanide. When the amount of sulfhydryl groups was determined by developing Prussian blue from ferrocyanide, the undesired action between ferricyanide ion and sugar could be compensated for by blanks containing sugar but no egg albumin. When the sulfhydryl groups were determined by using ferricyanide as a titrating agent with the end-point being indicated by a negative nitroprusside test, any reaction between ferricyanide ion and sugar occurring at the same time could not be measured. Thus, it was possible in experiments with guanidine hydrochloride as denaturing agent that the sugars could have prevented some denaturation but it was undetected because some ferricyanide reacted with sugar in a side reaction. It seems highly unlikely, however, that the reaction of ferricyanide ion with sugar would be exactly equivalent to the number of sulfhydryl groups protected by the sugar. This is indicated particularly when it is noted that fructose reacts more readily with ferricyanide ion than glucose but prevents about the same amount of denaturation. Yet in the presence of either sugar, titration for sulfhydryl groups in egg albumin was the same as for egg albumin alone. It was concluded, therefore, that sugars did not prevent egg albumin denaturation brought about by guanidine hydrochloride.

Exhaustive experimentation failed to demonstrate any protective effect by glucose, fructose, galactose, or sucrose against denaturation brought about by sodium dodecyl sulfate even when the amount of denaturing agent was at a minimum and the concentration of sugar was as high as 1 M. Advantage was taken of this fact in the experiments with heat denaturation in the presence of sugar to show that the decrease in sulfhydryl groups measured was caused by inhibition of denaturation and not by air oxidation of these reactive groups. Thus aliquots of every egg albumin solution denatured by heat in the presence of sugar were subjected to further denaturation by sodium dodecyl sulfate. If the full number of sulfhydryl groups then could be detected in these aliquots it was a clear indication that no reactive groups had been lost through air oxidation in the denaturation procedure and subsequent handling. Therefore, any diminution of sulfhydryl groups detected in heat denatured egg albumin was the result of the protective effect of the sugar present at the time of denaturation, not the result of air oxidation of sulfhydryl groups during the denaturation procedure.

In the first heat denaturation experiments the procedure described by Anson (23) was carried out in an open test tube with occasional shaking during the heating period. Subsequent measurement of the sulfhydryl groups in a one ml. aliquot of the solution never revealed the full amount of sulfhydryl groups. This was unchanged by further denaturation by sodium dodecyl sulfate prior to the addition of ferricyanide, yet denaturation by sodium dodecyl sulfate of the starting egg albumin solution revealed the proper amount of sulfhydryl groups. It was concluded that some of the sulfhydryl groups were being destroyed by air oxidation during the heat denaturation process. This was indicated experimentally when the heat denaturation was performed in a Thunberg tube after the atmosphere had been replaced with oxygen-free nitrogen. The full number of sulfhydryl groups was detected regularly when this technique was used. All subsequent heat denaturation experiments with sugars present were performed in Thunberg tubes following the latter technique.

Hardt (4) showed that the length of time (a few minutes to 96 hours) that sugar was in contact with egg albumin had no influence on the amount of protection against heat denaturation. This effect was indicated in a negative way when sodium dodecyl sulfate was used as denaturant. No protection was shown whether sugar was present for a few minutes or twenty-four hours before egg albumin was denatured. When egg albumin was denatured by heat and acid at 50°C. the effect was very evident. No difference was detected whether the egg albumin was in contact with sugar for some time before acid was added or whether the sugar and acid were mixed together and thus added at the same time that the heating was begun. Thus, the protective action is shown to take place almost instantaneously even in a very acid solution (pH 1.0 or less, approx. 0.14 N HCl).

Although one of the principal criteria of denaturation traditionally has been precipitation at the isoelectric point, the processes (denaturation and precipitation) are separable. In the work of Duddles (3), which depended on coagulation of egg albumin at the isoelectric point (pH 4.8) as a quantitative measure of denaturation, it was a question whether sugars prevented heat denaturation or prevented the precipitation of otherwise denatured egg albumin. Since egg albumin heated in the presence of sugar did not precipitate when the sugar was dialyzed out of the solution, the evidence indicated that sugars did prevent denaturation. Hardt (4) utilized another criterion of denaturation, the appearance of sulfhydryl groups, to indicate even more definitely that sugars prevent heat denaturation both at pH 4.8 and pH 8.6. The appearance of sulfhydryl groups is one of the earliest detectable changes in the denaturation of egg albumin and therefore is a more unequivocal indication of denaturation than is coagulation. The methods for measuring sulfhydryl groups utilised by Hardt indicated only half the sulfhydryl groups that Anson (9) later showed to be present. Utilizing a method developed by Anson which reveals all the sulfhydryl groups in egg albumin, the present work has demonstrated that in the presence of 1 M concentration of several sugars at pH 1.0 only about seventy per cent of the available sulfhydryl groups are revealed. Thus, the present work and previous work in this laboratory have shown that the protective action of sugars against heat denaturation of egg albumin is demonstrable from pH 1.0 to pH 8.6 at temperatures for any specific pH which are sufficient to completely denature the protein in the absence of sugar.

That there is an association between sugars and egg albumin which stabilizes the native molecule toward heat seems likely. Such an association has not been demonstrated directly, however, and if there is one it is sufficiently weak so that it cannot be demonstrated in the presence of twelve mg. of sodium dodecyl sulfate, the minimum amount necessary to bring about complete denaturation of egg albumin. Neither can an association between sugar and egg albumin be demonstrated in the presence of six M guanidine hydrochloride.

It is not surprising, perhaps, that sugars are not effective inhibitors of denaturation by guanidine hydrochloride when it is noted that fatty acid anions (particularly C_7 and C_8 chains) which were indicated by Boyer, Ballou, Luck and coworkers to have considerable stabilizing action in low concentration against heat denaturation of serum albumin (13) were completely ineffective in the presence of 6 M guanidine hydrochloride (14). Further, Boyer, *et al.* (13) found that sugars and alcohols were among compounds which had only slight stabilizing properties for serum albumin against heat. On the other hand, preliminary experiments showed that although egg albumin was protected in some degree by caprylate in low concentrations other proteins were rendered more heat labile in the presence of caprylate.

The work of Luck *et al.* (13, 15, 16) may partially explain why sugars are ineffective against the denaturing action of sodium dodecyl sulfate. Their experiments showed that at very low concentrations the effectiveness of fatty acid anions as stabilizers of serum albumin increased as the chain length increased up to C_{12}. They were able to show that this effectiveness was paralled by an increased affinity of the longer chain fatty acid anion for serum albumin, and at higher concentrations, where the maximum stabilisation is produced by C_7 and C_8 acids, the protective effect of sodium dodecyl sulfate has been supplanted by its denaturing action and ability to keep proteins in solution. The affinity of long chain fatty acid anions for proteins appears to be due to both the negative charge and the non-polar chain and is so great that it cannot be replaced in any noticeable way by the relatively weak non-polar affinity of the sugars.

EXPERIMENTAL - PART TWO

EXPERIMENTAL - PART TWO

Since the present investigation and previous work have indicated that the inhibition to denaturation by sugars occurred over a wide pH range and required only a short period of exposure to the specific sugar, it seemed possible that there was a definite combination between egg albumin and the sugar. It was thought that such a definite combination might have electrophoretic properties different from those of native egg albumin. Several investigators (17, 18) already have shown that a number of detergents show definite complex formation which can be demonstrated electrophoretically with various proteins including egg albumin. These detergents are charged molecules but the complexes have electrophoretic mobilities in between that of native egg albumin and that of pure detergent. Since such was the case it seemed reasonable to suppose that a definite combination between egg albumin and sugar might result in a slower moving fraction, since sugars are not charged and do not migrate in an electric field.

MATERIALS

The chemical reagents and the egg albumin used in the experiments described in this section were the same as those described in Experimental-Part One.

SOLUTIONS FOR ELECTROPHORESIS

Native egg albumin, mixtures of egg albumin and a sugar, and egg albumin heated either alone or with a sugar or related compound at 50°C. for various periods of time were examined electrophoretically at one or more of three different pH levels. The pH levels and buffer systems were: pH 5.1, 0.034 M acetic acid-0.1 M sodium acetate; pH 6.8, 0.025 M monosodium phosphate-0.025 M disodium phosphate; pH 3.0, 0.5 M glycine-0.1 M hydrochloric acid. Ionic strength was kept constant at 0.1 and all experiments were performed at a temperature of 1°C.

In those experiments in which egg albumin was heated for various periods of time with a sugar, the concentration of sugar indicated is that present at the time of heating. Except for those experiments marked, no dialysis (see below), the amount of sugar present during the electrophoretic analysis was negligible.

Approximately two ml. of egg albumin mixture was usually diluted to ten ml. with buffer before dialysis was begun. The protein solution was then dialyzed at room temperature against 100 ml. of buffer for one and one-half hours followed by dialysis against two fresh 100 ml. portions of buffer for three hours each. The dialysis was carried out in a cellophane bag made from one inch tubing (Visking Corp.) with either the bag or the buffer container turning slowly. Finally 200 ml. of buffer was added to the third 100 ml. portion of buffer and the dialysis continued overnight in the cold room (3-5°C.) without stirring. This buffer was then used to fill the electrode vessels. The conductivity of both the buffer and the protein solution were measured and found to be approximately the same.

Although a definite combination between egg albumin and a sugar might exist in the presence of an excess of the sugar, such a combination might decompose if the excess sugar were removed. Since sugars readily dialyze, the protein-sugar mixtures in some experiments analyzed electrophoretically at pH 5.1 and pH 6.8 were not dialyzed. These mixtures were made with buffer of twice the usual concentration so that the final solution had an ionic strength of 0.1. These experiments are indicated by the notation, no dialysis.

No nitrogen analysis was made on the final protein solutions which were examined electrophoretically at pH 5.1 and pH 6.8. Since the concentration and volume of the initial egg albumin solution and the volume of the final solution used for electrophoresis were known, the approximate protein concentration could be estimated. The protein in one milliliter aliquots of the final solution examined electrophoretically at pH 3.0 was precipitated by the use of one milliliter of twenty per cent trichloracetic acid. After thorough washing with ten per cent trichloracetic acid the precipitate was analyzed for nitrogen by a semi-micro Kjeldahl method (22) and from these results, the protein concentration was calculated. Since the buffer at pH 3.0 contained a relatively large amount of glycine, it was necessary to separate the protein from the buffer before making a nitrogen analysis to determine protein concentration.

The Molisch test using thymol (29) was made on several solutions as a qualitative test for the presence of reducing sugar.

ELECTROPHORESIS APPARATUS

The electrophoresis apparatus used in these experiments was an analytical moving-boundary instrument, Model 38, made by the Perkin-Elmer Corporation. This instrument contained the usual three section electrophoresis cell with 2 x 15 x 50 mm. channels which required about two milliliters of protein solution. The schlieren scanning system of Longsworth (30) was used to record the moving boundaries. Both electrode vessels were open to the atmosphere. A concise description of the general experimental procedure is given by Alberty (31).

The apparatus was new when the experiments at pH 5.1 and pH 6.8 were made and a conductance cell capable of being used with the small quantities of protein needed for the electrophoresis cell was not yet available. Since the conductance of these solutions was not measured neither the electric field strength nor the mobility of the boundaries could be calculated for experiments made at pH 5.1 or pH 6.8.

RESULTS - PART TWO

RESULTS - PART TWO

ELECTROPHORESIS at pH 5.1

Both Duddles (3) and Hardt (4) have shown that sugars protect egg albumin against heat denaturation at pH 4.8, the isoelectric point of egg albumin. Therefore the first attempt to demonstrate a definite combination between egg albumin and sugars by electrophoretic analysis was carried out at a pH near the isoelectric point, that is, pH 5.1. Figure I shows comparable patterns of egg albumin alone, a mixture of egg albumin and 0.1 M glucose and 0.1 M glucose alone. The concentration of sugar was that present during electrophoresis. Although the patterns show the usual small inhomogenity of crystalline egg albumin, the only new peak is one caused by the diffusion of glucose at the initial boundary. The patterns give no indication of a combination between egg albumin and glucose which has a different electrophoretic mobility from egg albumin alone.

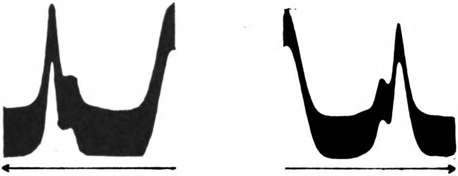
ELECTROPHORESIS at pH 6.8

It is well known that two protein components which are electrically inseparable at one pH can often be separated at another pH. The next attempt to demonstrate a definite combination between egg albumin and sugars by electrophoretic analysis was carried out at

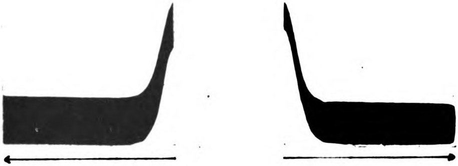
Figure 1. Electrophoretic Patterns of Native Egg Albumin, Egg Albumin with Glucose and Glucose Only. (pH 5.1; 0.1 M acetate buffer; $\Gamma/2 = 0.1$)

Ascending Descending

Native Egg Albumin. (18,000 secs.; conc. ca. 1%)



Native Egg Albumin in 0.1 M Glucose. No Dialysis. (15,183 secs.; conc. ca. 1.0%)



Glucose Only, 0.1 M. No Dialysis. (15,183 secs.)

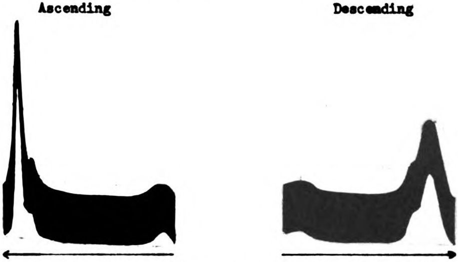
pH 6.8. Figure 2 shows comparable patterns of egg albumin alone, a mixture of egg albumin and 0.1 M glucose, and a mixture of egg albumin and 0.1 M glucose heated at 50°C. for six hours. The amount of sugar given was that present during electrophoresis. Again the small inhomogenity of crystalline egg albumin at intermediate pH values and the peak caused by the diffusion of glucose at the initial boundary is shown. No new peak indicating a combination between egg albumin and glucose is visible, however. Figure 3 shows comparable patterns of egg albumin heated at 50°C. for six hours with 0.1 M glucose at pH 6.8, with 2.0 M glucose at pH 3.15 and 1.0 M sucrose at pH 6.8. The amounts of sugar indicated were present at the time of heating, no sugar was present during electrophoresis. Again the patterns are like native egg albumin, alone.

ELECTROPHORESIS at pH 3.0

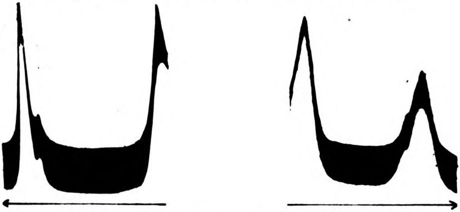
1. Components Produced by Heat.

Since electrophoresis at two pH values on the alkaline side of the isoelectric point of egg albumin showed no separation of a possible sugar-protein combination the next experiments were made at pH 3.0. Figure 4 shows patterns of native egg albumin, egg albumin, pH 3.0, heated alone and with 2.0 M glucose for twenty minutes at 50°C. Figure 5 shows patterns of egg albumin, pH 3.0, heated with 2.0 M glucose for one and one-half hour, three, and six

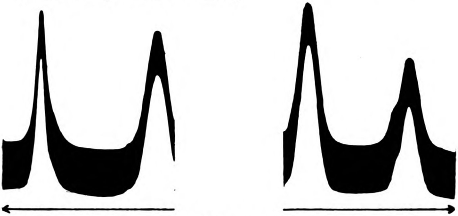
Figure 2. Electrophoretic Patterns of Native Egg Albumin, Egg Albumin with Glucose, Egg Albumin Heated with Glucose. (pH 6.8; 0.025 M phosphate buffer; $\Gamma/2 = 0.1$)



Native Egg Albumin. (7000 secs.; conc. ca. 1.0%)

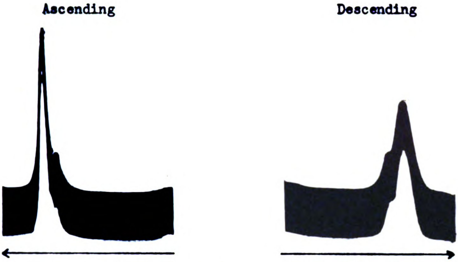


Native Egg Albumin in 0.1 M Glucose. No Dialysis. (6300 secs.; conc. ca. 1.0%)

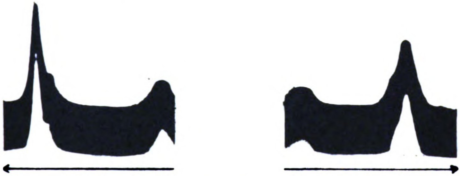


Egg Albumin Heated at 50°C. for 6 Hrs. in 0.1 M Glucose. No Dialysis. (5300 secs.; conc. ca. 1.0%)

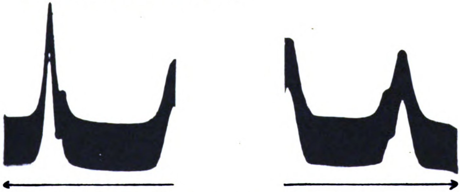
Figure 3. Electrophoretic Patterns of Egg Albumin Heated at 50°C. for Six Hours in the Presence of Glucose and Sucrose at Different pH Values.
(pH 6.8; 0.025 phosphate buffer; $\Gamma/2 = 0.1$)



Egg Albumin Heated at pH 6.8 in 0.1 M Glucose.
(6000 secs.; conc. ca. 1.0%)



Egg Albumin Heated at pH 3.15 in 2.0 M Glucose.
(6000 secs.; conc. ca. 1.0%)

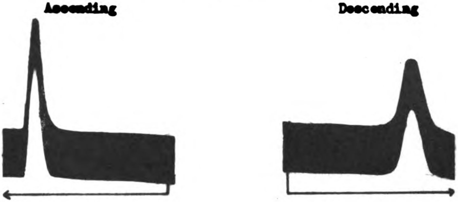


Egg Albumin Heated at pH 6.8 in 1.0 M Sucrose.
(6000 secs.; conc. less than 1.0%)

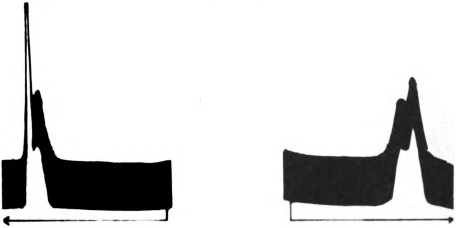
hours at 50°C. Figure 6 shows patterns of egg albumin, pH 3.0, heated with 2.0 M glucose for twenty-four hours at 50°C. and the supernatant and precipitate of such a mixture after adjustment to pH 4.8. These patterns indicated that heating egg albumin, pH 3.0, at 50°C., whether alone or with 2.0 M glucose, resulted in two components. The presence of glucose, however, altered the quantity of the two components produced. Thus egg albumin heated alone for twenty minutes resulted in a high fast moving peak and a low slow moving peak. Egg albumin heated with 2.0 M glucose for twenty minutes resulted in a high slow moving peak. As the period of heating was increased the height of the fast moving peak increased until at twenty-four hours the patterns were much like those of egg albumin heated alone for twenty minutes. Although the fast moving component appeared to have the same mobility as native egg albumin, separation of the components of a heated egg albumin-glucose mixture by adjustment to pH 4.8 showed that the fast moving component is the precipitable component and, therefore, is denatured egg albumin. The slower component remained in solution at pH 4.8 and presumably is native egg albumin. Table I gives the mobility of the components shown in Figures 4, 5 and 6.

The protein solutions which yielded the electrophoretic patterns given in Figures 4, 5 and 6 were tested for the presence of sugar by the qualitative Molisch test (29). The results for native egg albumin and egg albumin heated with 2.0 M glucose for one and one-half

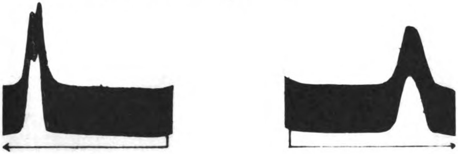
Figure 4. Electrophoretic Patterns of Native Egg Albumin, Egg Albumin Heated at 50°C. for Twenty Minutes, Alone and with Glucose.
(pH 3.0; 0.5 M glycine-0.1 M HCl buffer; $\Gamma/2 = 0.1$)



Native Egg Albumin.
(9000 secs.; 6.31 volts/cm.; conc. 0.86%)



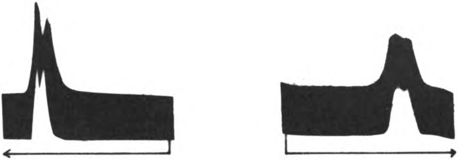
Egg Albumin Heated at pH 3.0.
(9000 secs.; 6.30 volts/cm.; 1.09%)



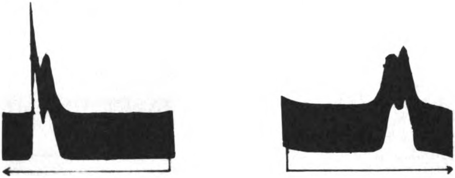
Egg Albumin Heated at pH 3.0 in 2.0 M Glucose.
(9000 secs.; 6.27 volts/cm.; conc. 0.76%)

Figure 5. Electrophoretic Patterns of Egg Albumin Heated at 50°C. for Various Times in 2.0 M Glucose.
(pH 3.0; 0.5 M glycine-0.1 M HCl buffer; $\Gamma/2 = 0.1$)

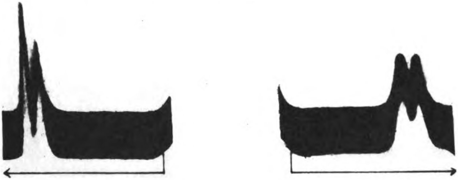
Ascending Descending.



Ninety Minute Heating at pH 3.0.
(9000 secs.; 6.30 volts/cm.; conc. 0.79%)



Three Hour Heating at pH 3.0.
(9000 secs.; 6.27 volts/cm.; conc. 0.76%)



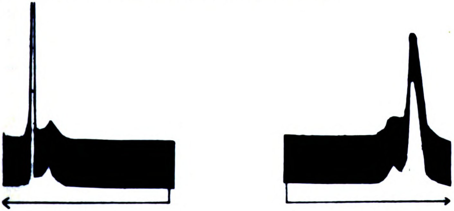
Six Hour Heating at pH 3.0.
(9000 secs.; 6.42 volts/cm.; conc. 0.97%)

Figure 6. Electrophoretic Patterns of Egg Albumin Heated For Twenty-four Hours at 50°C., pH 3.0, in 2.0 M Glucose, and of the Supernatant and Precipitate Resulting Upon Adjustment to pH 4.8.
(pH 3.0; 0.5 M glycine-0.1 M HCl buffer; $\Gamma/2 = 0.1$)

Ascending Descending

Twenty-four Hour Heating at pH 3.0.
(9000 secs.; 6.29 volts/cm.; conc. 0.76%)

Supernatant at pH 4.8.
(9000 secs.; 6.29 volts/cm.; conc. 0.32%)



Precipitate at pH 4.8.
(9000 secs.; 6.29 volts/cm.; conc. 1.03%)

TABLE I

THE MOBILITY OF THE COMPONENTS SHOWN IN FIGURES 4, 5 AND 6.

Material & Treatment	Mobility ($cm^2 \cdot volt^{-1} \cdot sec.^{-1} \cdot 10^{-5}$)			
	Ascending		Descending	
	Slow	Fast	Slow	Fast
Native Egg Albumin		+6.30		+5.83
Heated 20 Min.	+6.00	+6.38	+5.29	+5.82
2.0 M Glucose Heated 20 Min.	6.12	6.47		5.73
2.0 M Glucose Heated 90 Min.	5.73	6.17	5.38	5.87
2.0 M Glucose Heated 3 Hours	5.50	6.12	3.95	5.14
2.0 M Glucose Heated 6 Hours	5.73	6.38	5.45	6.19
2.0 M Glucose Heated 24 Hours	5.65	6.40	5.39	6.18
Supernatant	5.69		5.48	
Precipitate	5.47	6.36	5.12	6.00

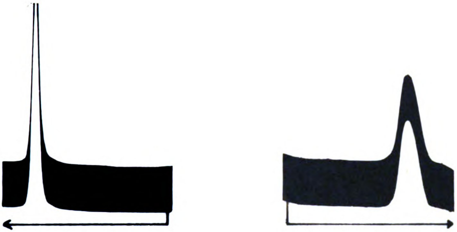
hour and twenty-four hours were negative. Egg albumin heated with 2.0 M glucose for twenty minutes, three hours, and six hours gave a trace test. The last buffer solutions against which these protein solutions had been finally dialyzed all gave negative tests. These results indicated that a protein-glucose combination stable to dialysis was not formed.

2. Separation and Study of Components Produced by Heat.

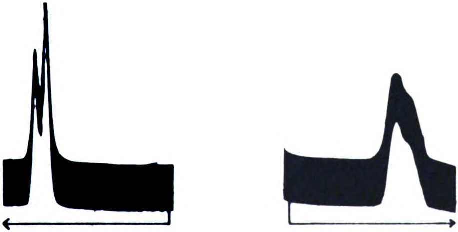
To indicate more definitely whether the two components found when egg albumin was heated alone were the same as the two components found when egg albumin was heated with 2.0 M glucose, the electrophoretic runs shown in Figures 7-10 were made. The mobility of the components found in these runs is given in Table II. Figure 7 shows patterns of native egg albumin and egg albumin, pH 3.0, heated at 50°C. for twenty minutes. Figure 8 shows patterns of the precipitate and supernatant resulting when the heated egg albumin was adjusted to pH 4.8. These patterns together with the mobilities given in Table II show that when egg albumin, pH 3.0, is heated at 50°C. the resulting fast component is precipitated at pH 4.8 and therefore, is denatured egg albumin, while the slow component is soluble at pH 4.8 and presumably is native egg albumin. This is true in spite of the fact that the precipitable fast component has the same mobility as the initial native egg albumin while the soluble slow component moves more slowly than the starting material. Figure 9 shows patterns of egg albumin, pH 3.0, heated with 2.0 M glucose at 50°C. for six hours

Figure 7. Electrophoretic Patterns of Native Egg Albumin, and Egg Albumin Heated for 20 Minutes at 50°C., pH 3.0. (pH 3.0; 0.5 M glycine-0.1 M HCl buffer; $\Gamma/2 = 0.1$)

Ascending Descending



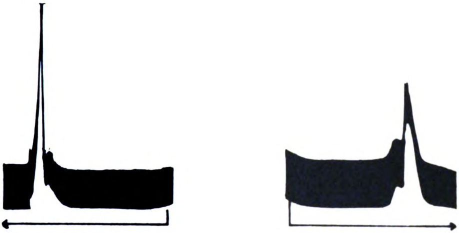
Native Egg Albumin Without Heat.
(9000 secs.; 6.40 volts/cm.; conc. 0.93%)



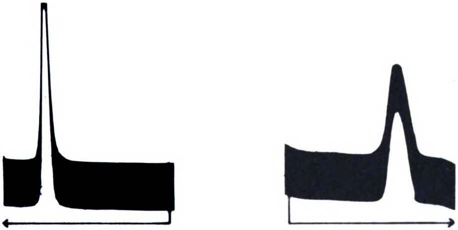
Egg Albumin After Heating.
(9000 secs.; 6.35 volts/cm.; conc. 1.27%)

Figure 8. Electrophoretic Patterns of the Precipitate and of the Supernatant After Adjustment to pH 4.8 of Egg Albumin, Heated for 20 Minutes at 50°C., pH 3.0.
(pH 3.0; 0.5 M glycine-0.1 M HCl buffer; $\Gamma/2 = 0.1$)

Ascending Descending

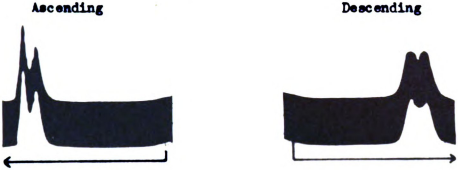


Precipitate at 4.8.
(9000 secs.; 6.34 volts/cm.; conc. 0.64%)

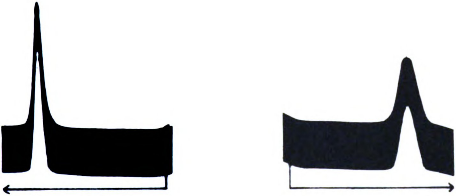


Supernatant at pH 4.8.
(9000 secs.; 6.39 volts/cm.; conc. 1.07%)

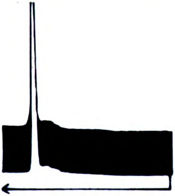
Figure 9. Electrophoretic Patterns of Egg Albumin Heated For Six Hours at 50°C., pH 3.0, in 2.0 M Glucose, and of the Supernatant and Precipitate Resulting Upon Adjustment to pH 4.8.
(pH 3.0; 0.5 M glycine-0.1 M HCl buffer; Γ/2 = 0.1)



Egg Albumin and Glucose.
(9000 secs.; 6.22 volts/cm.; conc. ca. 0.6%)



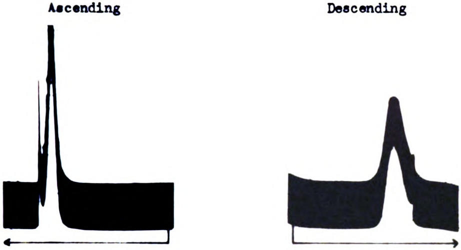
Supernatant at pH 4.8.
(9000 secs.; 6.40 volts/cm.; conc. 0.66%)



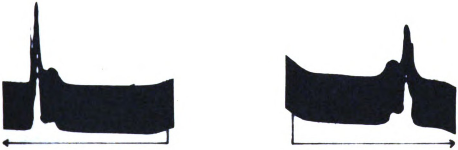
Not Recorded

Precipitate at pH 4.8.
(9000 secs.; 6.40 volts/cm.; conc. 0.83%)

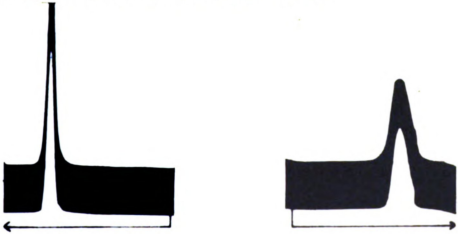
Figure 10. Electrophoretic Patterns of Egg Albumin Heated For Six Hours at 50°C., pH 3.0, in 2.0 M Glucose, and of the Supernatant and Precipitate Resulting Upon Adjustment to pH 4.8.
(pH 3.0; 0.5 M glycine–0.1 M HCl buffer; $\Gamma/2 = 0.1$)



Egg Albumin and Glucose.
(9000 secs.; 6.33 volts/cm.; conc. 0.92%)



Precipitate at pH 4.8.
(9000 secs.; 6.06 volts/cm.; conc. 0.36%)



Supernatant at pH 4.8.
(9000 secs.; 6.38 volts/cm.; conc. 0.81%)

TABLE II

THE MOBILITY OF THE COMPONENTS SHOWN IN FIGURES 7-10.

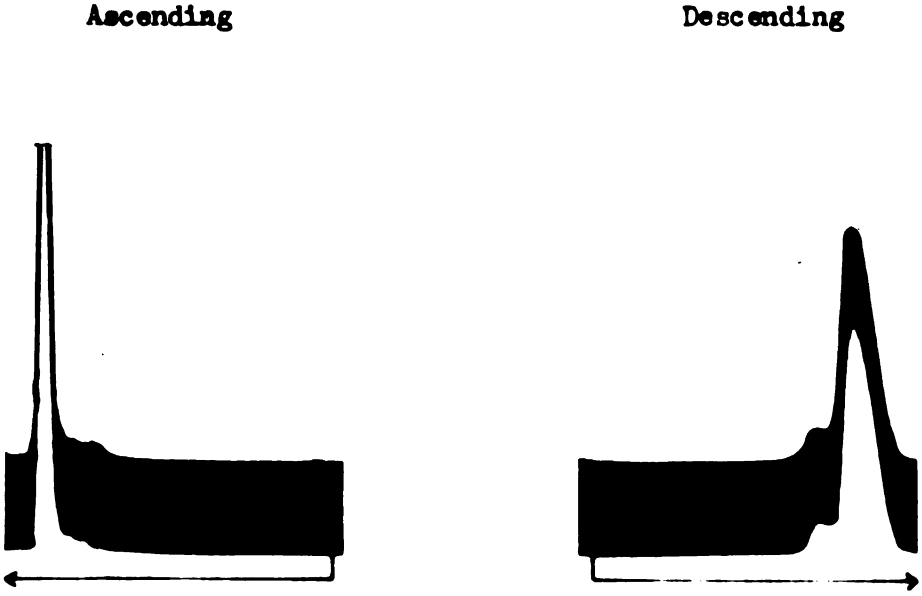
Material & Treatment	Mobility $(cm^2 \cdot volt^{-1} \cdot sec.^{-1} \cdot 10^{-5})$			
	Ascending		Descending	
	Slow	Fast	Slow	Fast
Native Egg Albumin		+6.12		+5.75
Heated 20 Min.	+5.65	6.16	+5.02	5.57
Precipitate	5.59	6.06	4.93	5.49
Supernatant	5.69		5.05	5.93
2.0 M Glucose Heated 6 Hours	6.14	6.68	5.66	6.32
Supernatant	6.02		5.60	
Precipitate		6.40		
2.0 M Glucose Heated 6 Hours	5.45	6.06	4.71	5.62
Precipitate	5.59	6.33	5.04	5.72
Supernatant	5.70		4.80	

and of the supernatant and precipitate resulting when this mixture was adjusted to pH 4.8. Again the fast component is precipitated at the isoelectric point of egg albumin while the slow component remains in solution. Because the mobilities of the components shown in Figure 9 are different from those shown in Figures 7 and 8, a second electrophoretic pattern of egg albumin, pH 3.0, heated with 2.0 M glucose at 50°C. for six hours is given in Figure 10. The mobilities of the components shown in Figure 10 are approximately the same as the mobilities of the components shown in Figures 7 and 8. Although the mobilities from one electrophoretic run to another are not constant, the results given in Tables I and II show, in general, that the presence of glucose does not change the mobility of the two components resulting when egg albumin is heated.

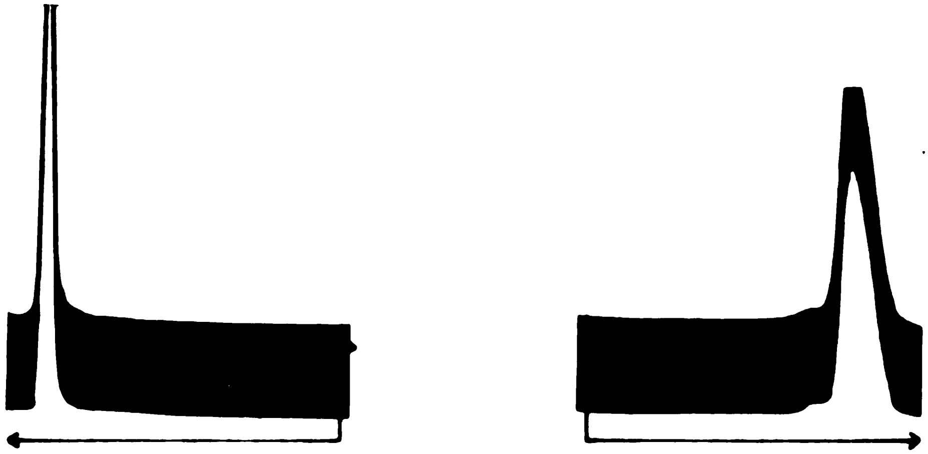
3. Effect of Two Sugars, Two Polyols and Glycine on the Two Components.

Figure 11 shows patterns of egg albumin alone, pH 3.0, heated at 50 C. for six hours and for twenty-four hours. Figure 12 shows patterns of egg albumin, pH 3.0, heated with 2.0 M glucose at 50°C. for six hours and for twenty-four hours. Figure 13 shows patterns of egg albumin, pH 3.0, heated with 2.0 M mannitol at 50°C. for six hours and for twenty-four hours. These results indicate that glucose and the somewhat related polyol, mannitol, have about the same protective effect for egg albumin against heat denaturation. Figure 14 shows patterns of egg albumin, pH 3.0, heated with 2.0 M. fructose at 50°C. for six hours and for twenty-four hours. These results show that fructose does not protect egg albumin against heat denaturation as well as glucose or mannitol. The patterns after heat for six hours with fructose are about the same as after heat for twenty-four hours with glucose. Figure 15 shows patterns of egg albumin, pH 3.0, heated with 2.0 M glycine at 50°C. for six hours and for twenty-four hours. These patterns show only one principal component and, since it is a slow moving component, this is taken as evidence that glycine completely protects egg albumin against denaturation under the circumstances of these experiments. Figure 16 shows patterns of egg albumin, pH 3.0, heated with 2.0 M glycerol at 50°C. for six hours and twenty-four hours. The patterns are almost identical to those shown in Figure 15 thus indicating that glycerol offers practically no protection against heat denaturation as carried out here. The mobility of the components shown in Figures 11-16 are given in Table III.

Figure 11. Electrophoretic Patterns of Egg Albumin Heated Alone for 6 and 24 Hours, at 50°C., pH 3.0. (pH 3.0; 0.5 M glycine–0.1 M HCl buffer; $\Gamma/2 = 0.1$)



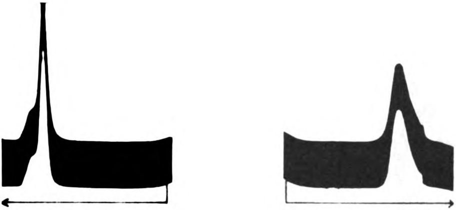
Six Hour Heating.
(9000 secs.; 6.43 volts/cm.; conc. 1.32%)



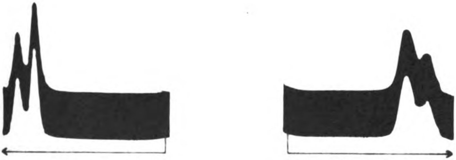
Twenty-four Hour Heating.
(9000 secs.; 6.41 volts/cm.; conc. 1.21%)

Figure 12. Electrophoretic Patterns of Egg Albumin Heated For Six and Twenty-four Hours at 50°C., pH 3.0, in the Presence of 2.0 M Glucose.
(pH 3.0; 0.5 M glycine-0.1 M HCl buffer; $\Gamma/2 = 0.1$)

Ascending Descending



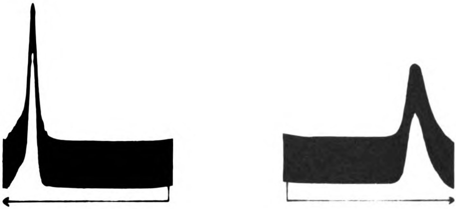
Six Hour Heating.
(9000 secs.; 6.35 volts/cm.; conc. 0.86%)



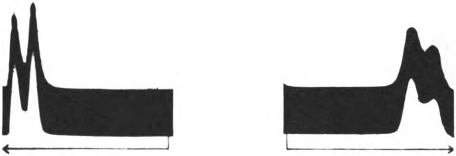
Twenty-four Hour Heating.
(9000 secs.; 6.38 volts/cm.; conc. 0.88%)

Figure 13. **Electrophoretic Patterns of Egg Albumin Heated For Six and Twenty-four Hours at 50°C., pH 3.0, in the Presence of 2.0 M Mannitol.**
(pH 3.0; 0.5 M glycine–0.1 M HCl buffer; $\Gamma/2 = 0.1$)

Ascending **Descending**



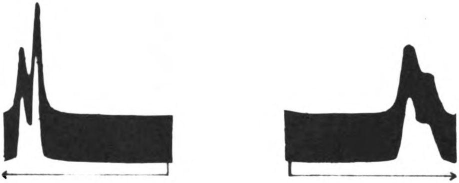
Six Hour Heating.
(9000 secs.; 6.38 volts/cm.; conc. 0.92%)



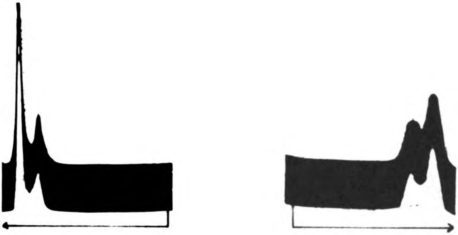
Twenty-four Hour Heating.
(9000 secs.; 6.40 volts/cm.; conc. 1.02%)

Figure 14. Electrophoretic Patterns of Egg Albumin Heated For Six and Twenty-four Hours at 50°C., pH 3.0, in the Presence of 2.0 M Fructose.
(pH 3.0; 0.5 M glycine—0.1 M HCl buffer; $\Gamma/2 = 0.1$)

Ascending Descending



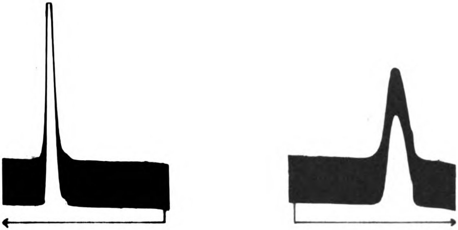
Six Hour Heating.
(9000 secs.; 6.41 volts/cm.; conc. 0.92%)



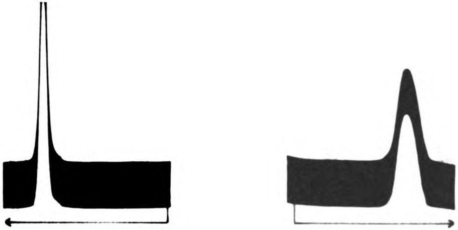
Twenty-four Hour Heating.
(9000 secs.; 6.35 volts/cm.; conc. ca. 1.0%)

Figure 15. Electrophoretic Patterns of Egg Albumin Heated For Six and Twenty-four Hours at 50°C., pH 3.0, in the Presence of 2.0 M Glycine.
(pH 3.0; 0.5 M glycine–0.1 M HCl buffer; $\Gamma/2 = 0.1$)

Ascending Descending



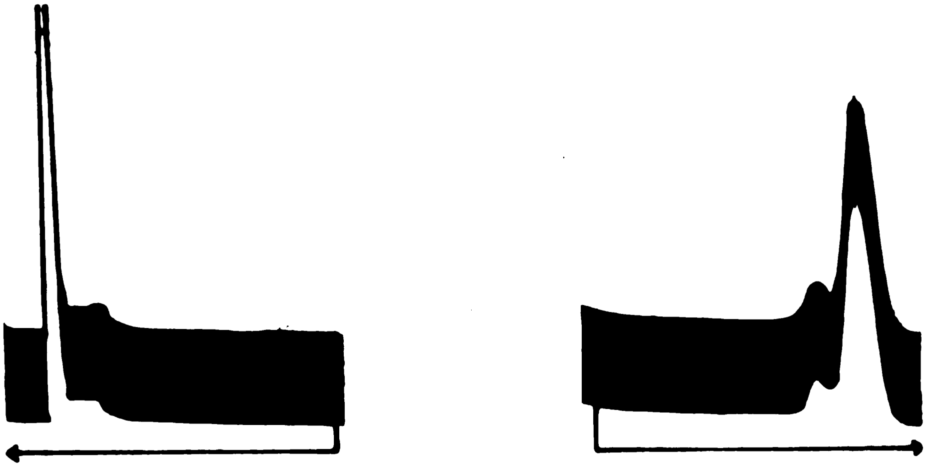
Six Hour Heating.
(9000 secs.; 6.38 volts/cm.; conc. 1.07%)



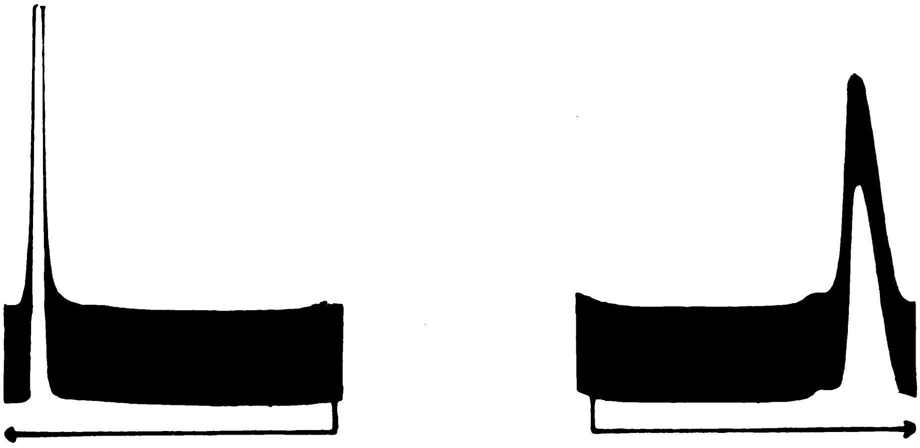
Twenty-four Hour Heating.
(9000 secs.; 6.38 volts/cm.; conc. 1.17%)

Figure 16. Electrophoretic Patterns of Egg Albumin Heated For Six and Twenty-four Hours at 50°C., pH 3.0, in the Presence of 2.0 M Glycerol.
(pH 3.0; 0.5 M glycine–0.1 M HCl buffer; Γ/2 = 0.1)

Ascending   Descending



Six Hour Heating.
(9000 secs.; 6.41 volts/cm.; conc. 1.20%)



Twenty-four Hour Heating.
(9000 secs.; 6.34 volts/cm.; conc. 1.24%)

TABLE III

THE MOBILITY OF THE COMPONENTS SHOWN IN FIGURES 11-16.

Material & Treatment	Mobility $(cm^2 \cdot volt^{-1} \cdot sec.^{-1} \cdot 10^{-5})$			
	Ascending		Descending	
	Slow	Fast	Slow	Fast
Egg Albumin Alone Heated 6 Hours	+5.70	+6.56	+5.27	+6.04
Egg Albumin Alone Heated 24 Hours		6.76		6.07
2.0 M Glucose Heated 6 Hours	5.81	6.30	5.29	6.27
2.0 M Glucose Heated 24 Hours	6.09	6.81	5.48	6.44
2.0 M Mannitol Heated 6 Hours		6.30		5.92
2.0 M Mannitol Heated 24 Hours	6.15	7.04	5.64	6.66
2.0 M Fructose Heated 6 Hours			5.56	6.47
2.0 M Fructose Heated 24 Hours	6.00	6.88	5.64	6.58
2.0 M Glycine Heated 6 Hours	4.70		4.58	
2.0 M Glycine Heated 24 Hours	5.92		5.31	
2.0 M Glycerol Heated 6 Hours	5.55	6.41	5.11	5.98
2.0 M Glycerol Heated 24 Hours		6.93	5.35	6.40

DISCUSSION - PART TWO

DISCUSSION - PART TWO

In all the runs made at pH 5.1 and pH 6.8 there was a small side boundary which was first pointed out by Longsworth (30) as occurring in crystalline egg albumin preparations at intermediate pH values. Longsworth, *et al*. (32) have shown that although there appeared to be some variability in the relative amount of the lesser component from preparation to preparation, it was found in all samples of egg albumin examined regardless of source or mode of preparation. Perlman (33) has indicated that the phosphorus content of the two components is different and that the mobility difference corresponds with this difference in phosphorus content. No other differences in properties of the two components were found.

Normally non-equilibration of the protein with the buffer by dialysis results in unstable boundaries or boundaries which do not correspond to protein components. One new boundary was thus formed at the initial boundary site by diffusion of sugar. This was readily indicated experimentally and such boundaries were thereafter ignored whenever undialyzed or incompletely dialyzed samples were used. No other changes were detected.

Because electrophoresis of a mixture of egg albumin and glucose did not reveal any new protein component either at pH 5.1 or pH 6.8, heat was applied to some mixtures with the hope of increasing the amount of the presumed egg albumin-sugar component. Since the protective effect of sugars had been demonstrated only against heat denaturation there was no experimental indication that an egg albumin-sugar combination existed except at higher temperatures. The use of heat did not alter the pattern and the conditions under which heat was applied were so mild that no or very little denaturation may have taken place. One of the runs was made with an egg albumin-glucose mixture heated at pH 3.0 and 50°C. for six hours. These conditions later were shown to cause denaturation detectable with electrophoresis at pH 3.0, but electrophoresis at pH 6.8 revealed no protein-sugar component or anything indicative of partial denaturation of the egg albumin. This result follows the experience of several other investigators (30, 32, 34, 35) who have shown the mobility of denatured egg albumin differs only slightly from that of native egg albumin.

Electrophoresis at pH 3.0 revealed only one component in native egg albumin following the experience of other investigators (32). After egg albumin had been heated at 50°C. and pH 3.0 two components were visible and the experimental results (Figures 7 and 8, Table II) show that the faster component is denatured egg albumin and the slower component is presumably native egg albumin, although its mobility is slower than that of the original native egg albumin. The presence of sugars obviously changes the quantity of these two components resulting from a given amount of heat, but does not alter their mobility. The experiments pictured in Figures 9 and 10 show that the faster component produced in the presence of sugars again is precipitated at the isoelectric point of egg albumin whereas the slower component remains in solution. Electrophoresis has thus become another means of indicating that sugars do protect egg albumin against heat denaturation for a time, although eventually the egg albumin is denatured. Hardt (36) previously had shown that sugars inhibit or prevent the formation of the "C" component which appears after heating bovine serum or plasma.

The mobility of the two components is not as uniform as one would like to be assured that they are the same components from one run to another. Part of this non-uniformity may be due to unknown experimental errors. Another explanation is indicated by MacPherson and Heidelberger (37) who encountered extreme difficulty in obtaining successive preparations of acid-denatured egg albumin with reproducible properties. Experimental substantiation of this explanation for the variation encountered is indicated when it is noted that the mobilities of the components shown in Figure 9 (see Table II) which came from one preparation are relatively uniform. This is true also of another similar preparation shown in Figure 10 (see Table II). The mobilities of the components of the two preparations are quite different although each was supposedly prepared in the same manner.

Protein denaturation in general is dependent in some degree upon hydrogen ion concentration. Steinhardt (38) first explained the influence of pH on the rate of protein denaturation as being the result of the effect on the ionization of critical groups. Luck, *et al*. (16) have indicated that the protective effect of fatty acid anions for serum albumin against heat is due, at least in part, to an affinity for the charged groups of the albumin, a condition detected electrophoretically. It was shown also that the non-polar portion of the molecule was important in the stabilizing action. Therefore it seemed probable that the stabilization was the result of the attraction of different groups of the albumin molecule for both the polar and non-polar portions of the fatty acid anions. Other investigators (17, 18) have shown a definite combination of detergents with the charged groups of protein materials. Again the non-polar portion of the molecule is important in the affinity of the compound for protein. Since these materials are relatively large molecules the view often is advanced that the affinity for protein is great enough to force the protein molecule apart at various points thus resulting in loosening of the protein structure and thereby causing denaturation. Because the charge state of a protein has been indicated to be important in the denaturation of the material, it seemed possible that the protective effect of sugars for egg albumin against heat might be due in part to an effect on the charged group which could be demonstrated electrophoretically. Since sugars appear to have no effect on the mobility of egg albumin at several different pH levels it must be concluded that the protective effect is dependent solely upon a combination with the non-polar side chains of the protein. Although such a combination has yet to be demonstrated, it seems reasonable, in view of the importance of the non-polar portions of fatty acid anions and detergents, to believe that one exists. Since only the non-polar portion of the protein apparently is involved in the protective action by sugars, their relatively low activity can be explained.

Putnam (39) has suggested that the protective effect of sugars may be due to the osmotic property of the added solute. However, earlier work in this laboratory (3, 4) showed substantial differences between different sugars at the same molar concentration. The present work has shown that glycerol is much less effective than glucose at the same molar concentration. Such evidence does not support Putnam's suggestion.

Previous workers (2, 3, 4) have indicated differences in the protective efficiency of several sugars. The present work has demonstrated smaller differences than were previously indicated, probably because of the lower pH at which the denaturation was performed. Both this and earlier work (2) indicate that glycerol is a relatively poor stabilizing agent. In fact of all the sugars and polyols tried in this laboratory (3, 4 and the present work) glycerol has been the poorest stabilizing agent. There appears to be relatively little difference in protective action between pentoses, hexoses, a hexitol, and a disaccharide. The apparently excellent stabilizing action of glycine probably resulted from its buffering action because in all cases, the protective compound was added to egg albumin adjusted to pH 3.0. Sugars and polyols which have no buffering action changed the pH only slightly. Addition of glycine probably shifted the pH upward from pH 3.0 to a pH where egg albumin is stable at 50°C.

CONCLUSIONS

CONCLUSIONS

1. The sugars, glucose or fructose, were shown not to have any protective action against egg albumin denaturation caused by 6 M guanidine hydrochloride.
2. The sugars, glucose, fructose, galactose or sucrose, were shown not to have any protective action against egg albumin denaturation caused by a minimum amount of sodium dodecyl sulfate.
3. The sugars, glucose, fructose, galactose or sucrose in a concentration of 1.0 M were all shown to have a substantial protective effect against heat denaturation of egg albumin in acid media (approximately 0.14 N hydrochloric acid). This protective effect occurs almost instantaneously.
4. No combination of glucose and egg albumin could be directly demonstrated by electrophoresis at pH 3.0, 5.1 or 6.8.
5. Egg albumin, pH 3.0, heated at 50°C., either alone or with sugar, resulted in two components; one component was shown to be denatured egg albumin while the other component was shown to be native egg albumin.
6. The stabilizing effect of glucose, fructose, mannitol and glycine on egg albumin held at pH 3.0 and 50°C. for six hours and twenty-four hours was demonstrated electrophoretically.
7. No stabilizing effect by glycerol under the same circumstances could be demonstrated.

LITERATURE CITED

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(1) Neuberg, C., Hydrotropische Erscheinungen I. Mitteilung, Biochem. Z., 76, 107 (1916).

(2) Beilinsson, A., Thermostabilisation der Eiweisslosungen mit Rohrzucker und Glycerin, Biochem. Z., 213, 399 (1929).

(3) Duddles, W.G., The Effect of Sugars and Mannitol Upon Coagulation of Egg Albumin, Thesis for the Degree of M. S., Michigan State College, East Lansing, Mich., (1932).

(4) Hardt, C.R., Sulfhydryl Groups and the Coagulation of Proteins as Influenced by Reducing Sugars, Thesis for the Degree of M. S., Michigan State College, East Lansing, Mich., (1941).

(5) Arnold, V., Eine Farvenreaktion von Eiweisskorpen mit Nitroprussidnatrium, Z. physiol. Chem., 70, 300 (1911).

(6) Rosner, L., The Reaction Between Iodoacetic Acid and Denatured Egg Albumin, J. Biol. Chem., 132, 657 (1940).

(7) Todrick, A. and Walker, E., Sulfhydryl Groups in Proteins, Biochem. J., 31, 292 (1937).

(8) Mirsky, A.E., and Anson, M.L., Sulfhydryl and Disulfide Groups of Proteins. I. Methods of Estimation, J. Gen. Physiol., 18, 307 (1934-35).

(9) Anson, M.L., The Reactions of Denatured Egg Albumin with Ferricyanide, J. Gen. Physiol., 23, 247 (1939-1940).

(10) Anson, M.L., The Sulfhydryl Groups of Egg Albumin, J. Biol. Chem., 135, 797 (1940).

(11) Anson, M.L., Personal Communication.

(12) Ballou, G.A., Boyer, P.D., Luck, J.M., and Lum, F.G., The Heat Coagulation of Human Serum Albumin, J. Biol. Chem., 153, 589 (1944).

(13) Boyer, P.D., Lum, F.G., Ballou, G.A., Luck, J.M., and Rice, R.G., The Combination of Fatty Acids and Related Compounds with Serum

Albumin. I. Stabilization Against Heat Denaturation, J. Biol. Chem., 162, 181 (1946).

(14) Boyer, P.D., Ballou, G.A., and Luck, J.M., The Combination of Fatty Acid and Related Compounds with Serum Albumin. II. Stabilization Against Urea and Guanidine Denaturation, J. Biol. Chem., 162, 199-208 (1946).

(15) Boyer, P.D., Ballou, G.A., and Luck, J.M., The Combination of Fatty Acids and Related Compounds with Serum Albumin. III. The Nature and Extent of the Combination, J. Biol. Chem., 167, 407 (1947).

(16) Ballou, G.A., Boyer, P.D., and Luck, J.M., The Electrophoretic Mobility of Human Serum Albumin as Affected by Lower Fatty Acid Salts, J. Biol. Chem., 159, 111 (1945).

(17) Lundgren, P., Elam, D.W., and O'Connell, R.A., Electrophoretic Study of the Action of Alkylbenzenesulfonate Detergents on Egg Albumin, J. Biol. Chem., 149, 183 (1943).

(18) Putnam, F.W., and Neurath, H., Interaction Between Proteins and Synthetic Detergents. II. Electrophoretic Analysis of Serum Albumin-Sodium Dodecyl Sulfate Mixtures, J. Biol. Chem., 159, 195 (1945).

(19) Anson, M.L., The Sulfhydryl Groups of Egg Albumin, J. Gen. Physiol., 24, 399 (1940-41).

(20) Kekwick, R.A., and Cannan, R.K., The Hydrogen Ion Dissociation Curve of the Crystalline Albumin of the Hen's Egg, Biochem. J., 30, 227 (1936).

(21) Changes in the Official and Tentative Methods of Analysis Made at the Fifty-sixth Annual Meeting, Oct. 28, 29, 30 (1940) XLI Microchemical Methods Microkjeldahl Nitrogen Method J. Assoc. Off. Agr. Chemists, 24, 97 (1941).

(22) Clark, E.P., Semimicro Quantitative Organic Analysis, Academic Press, Inc. New York, N.Y. (1943) Chap. 3 (p. 37-43).

(23) Murray, Benjamin L., Standards and Tests for Reagent and C.P. Chemicals, Second Edition, p. 379, D. Van Nostrand Company, Inc., New York, N.Y., (1927).

(24) Treadwell, F.P., and Hall, W.T., Analytical Chemistry Vol. II 8th Edition, p. 640, John Wiley & Sons, Inc., New York, N.Y., (1935).

(25) Folin, O., and Malmros, H., An Improved Form of Folin's Micro Method for Blood Sugar Determinations, J. Biol. Chem., 83, 115 (1929).

(26) Mirsky, A.E., Sulfhydryl Groups of Egg Albumin in Different Denaturing Agents, J. Gen. Physiol., 24, 709 (1940-1941).

(27) Anson, M.L., Protein Denaturation and the Properties of Protein Groups, Advances in Protein Chemistry, Vol. II, p. 361-386, Academic Press Inc., New York, N.Y., (1945).

(28) Anson, M.L., Some Factors Which Influence the Oxidation of Sulfhydryl Groups. J. Gen. Physiol., 25, 355 (1941-42).

(29) Hawk, P.B. Oser, B.L., and Summerson, W.H., Practical Physiological Chemistry, Twelfth Edition, p. 54, The Blakiston Co., Philadelphia, Toronto.

(30) Longsworth, L.G., A Modification of the Schlieren Method for Use in Electrophoretic Analysis, J. Am. Chem. Soc., 61, 529 (1939).

(31) Alberty, Robert A., Electrochemical Properties of the Proteins and Amino Acids, The Proteins, Vol. I, Part A, p 461-548, Academic Press Inc., New York, N.Y., (1953).

(32) Longsworth, L.G., Cannon, R.K., and MacInnes, D.A., An Electrophoretic Study of the Protein of Egg White, J. Am. Chem. Soc., 62, 2580-2590 (1940).

(33) Perlmann, G.E., Enzymatic Dephosphorylation of Ovalbumin and Plakalbumin, J. Gen. Physiol., 35, 711-26 (1952).

(34) Landsteiner, K., Longsworth, L.G., and van der Scheer, J., Electrophoresis Experiments with Egg Albumins and Hemoglobins, Science, 88, 83-5 (1938).

(35) MacPherson, Catherine F.C., Heidelberger, M., and Moore, D.H., Denatured Egg Albumin. II. Viscosities, Particle Weights, and Electrophoretic Mobilities of Crystalline Egg Albumin Denatured in Various Ways, J. Am. Chem. Soc., 67, 578 (1945).

(36) Hardt, C.R., Huddleson, I.F., and Ball, C.D., An Electrophoretic Analysis of Changes Produced in Blood Serum and Plasma Proteins by Heat in the Presence of Sugars, J. Biol. Chem., 163, 211 (1946).

(37) MacPherson, Catherine F.C., and Heidelberger, M., Denatured Egg Albumin I. The Preparation and Purification of Crystalline Egg Albumin Denatured in Various Ways, J. Am. Chem. Soc., 67, 574 (1945).

(38) Steinhardt, Jacinto, The Stability of Crystalline Pepsin, Kgl. Danske Videnskab. Selskab., Mat.-fys. Medd. 14, No. 11 (1937).

(39) Putnam, Frank W., Protein Denaturation, The Proteins, Vol. I, Part A, p. 807, Academic Press Inc., New York, N.Y., (1953).