THE INFLUENCE OF SUGARS ON THE FORMATION OF SULFHYDRYL GROUPS IN HEAT DENATURATION AND HEAT COAGULATION OF EGG ALBUMIN

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Beilinsson (1) in 1929 showed that sucrose and glycerol inhibited the heat coagulation of egg albumin. Bancroft and Rutzler (2) claimed that the sugar peptized the coagulated protein. Harris (3) by the use of the nitroprusside reaction showed that sulfhydryl groups were not present in raw egg white but their presence could be demonstrated after heat coagulation. Various workers (4-7) have reported the formation of compounds between sugars and amino acids, simple peptides, and, in some cases, between sugars and proteins. Schubert (8) prepared crystalline compounds of a number of sugars with cysteine, compounds in which the nitroprusside test was negative. Von Przylecki and Cichocka (9) formed compounds which they called "symplexes" with maltose and several proteins. The "symplexes," formed at pH values between 7 and 10, were believed to involve the lysine residues of the proteins. No such compound was formed with sucrose.

In the present work, the protective action of sugars toward coagulation was thought to be due to the influence on the sulfhydryl groups in the denaturation phase of the process. To obtain evidence on this point, egg albumin solutions were heated in the presence and in the absence of sugars, and the sulfhydryl content of the egg albumin was determined by two different methods. The inhibition of heat coagulation exerted by sugars has also been shown by determining the amount of uncoagulated nitrogen when samples of egg albumin were heated in the presence and absence of sugars.

To indicate whether "symplexes," similar to those described by von Przylecki and Cichocka, were involved in this protective action, samples of egg albumin were allowed to stand at 5° for 96 hours at pH 8.6 and also at pH 4.8. After adjustment to the same pH, the amount of uncoagulated nitrogen was determined in each case. A coagulated "symplex," prepared similarly to that described by the above workers, has also been prepared and the amount of reducing substances present in this "symplex" compared with that present in ordinary heat-coagulated egg albumin.

EXPERIMENTAL

The experimental work was divided into three parts: (1) the influence of sugars and mannitol on the liberation of sulfhydryl groups; (2) the influ-
ence of sugars on the heat coagulation of egg albumin; (3) an attempt to show protein-carbohydrate combination.

Egg albumin was prepared by the method of Kekwick and Cannan (10). Dialysis of the sodium sulfate was carried out under reduced pressure. The nitrogen content of the egg albumin solution was determined by a micro-Kjeldahl method (11).

Two methods were used to determine the sulfhydryl groups, neither of which involved the probability of denaturation of the protein prior to the determination. The first of these methods involved the use of iodoacetic acid as described by Rosner (12); the second utilized the phenolindo-2,6-dichlorophenol titration as described by Todrick and Walker (13).

For the iodoacetic acid method, standard solutions of recrystallized cysteine hydrochloride containing the equivalent of 0.07 to 0.35 mg. of cysteine per ml. were prepared. To 1.5 ml. portions of these solutions were added 4.5 ml. of distilled water. The mixture was heated at 70° for 15 minutes and then allowed to cool. 2 ml. of a phosphate buffer, pH 7.4, and 2 ml. of 0.1 N iodoacetic acid, previously neutralized to pH 7.4, were added, and the mixture was allowed to stand for 30 minutes. At the end of that time, 0.25 ml. of a solution of 10 per cent trichloroacetic acid by weight and 0.25 ml. of a solution of 10 per cent sulfuric acid by weight were added and allowed to stand for 5 minutes and the solution was then filtered. To the filtrate was added 0.25 ml. of 3 per cent hydrogen peroxide by weight and the mixture was made up to 10 ml. with distilled water. After it had stood for 35 minutes, the color developed was determined by use of a photometer with a blue filter having a maximum absorption range of 4500 to 5000 Å. The standardization repeated with solutions of d-glucose in place of distilled water gave identical results with those obtained without glucose. The egg albumin solution was adjusted to pH 4.8 with 0.1 N hydrochloric acid. 1.5 ml. aliquots, containing 40.62 mg. of egg albumin per ml., were pipetted into test-tubes, and 4.5 ml. of the sugar solution or distilled water added. These mixtures were then treated in exactly the same manner as described in the standardization procedure. The mg. of cysteine were read from the standardization curve. The results with d-glucose, d-fructose, d-mannose, L-arabinose, d-xylose, and the hexatomic alcohol d-mannitol are given in Table I. Mannitol was added to this group of sugars to compare the effect of the absence of a carbonyl group. Each figure given in Table I represents the average of from seven to ten determinations. Results in the table have been corrected for blanks on the reagents. The addition of unheated egg albumin to these blanks did not in any case change their value.

For the titration method with phenolindo-2,6-dichlorophenol, cysteine hydrochloride was also used for standardization. A weighed amount of the recrystallized cysteine hydrochloride was first neutralized in an atmosphere
of nitrogen, dissolved in 2 ml. of a phosphate buffer, pH 4.8, and made up to 10 ml. with freshly boiled distilled water. The solution was then titrated in an atmosphere of nitrogen with a 0.1 per cent solution of phenolindo-2,6-dichlorophenol. 0.1 mg. of cysteine was found to be equivalent to 3 ml. of the phenolindo-2,6-dichlorophenol. 1.5 ml. aliquots of an egg albumin solution, containing 40.62 mg. of egg albumin per ml., were then pipetted into test-tubes. To each of these were added 4.5 ml. of distilled water, or sugar solution, 2 ml. of buffer, pH 4.8, and 2 ml. of distilled water.

Table I
Influence of Inhibiting Substances on Apparent Cysteine Content of Heat-Denatured Egg Albumin

<table>
<thead>
<tr>
<th>Inhibiting substance</th>
<th>Concentration</th>
<th>Cysteine in 60.93 mg. egg albumin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Iodoacetic acid method</td>
</tr>
<tr>
<td></td>
<td></td>
<td>mg.</td>
</tr>
<tr>
<td>Water</td>
<td>0.045</td>
<td>0.359</td>
</tr>
<tr>
<td>d-Glucose</td>
<td>0.225</td>
<td>0.289</td>
</tr>
<tr>
<td></td>
<td>0.450</td>
<td>0.220</td>
</tr>
<tr>
<td>d-Fructose</td>
<td>0.045</td>
<td>0.347</td>
</tr>
<tr>
<td></td>
<td>0.225</td>
<td>0.296</td>
</tr>
<tr>
<td></td>
<td>0.450</td>
<td>0.251</td>
</tr>
<tr>
<td>d-Mannose</td>
<td>0.045</td>
<td>0.353</td>
</tr>
<tr>
<td></td>
<td>0.225</td>
<td>0.310</td>
</tr>
<tr>
<td></td>
<td>0.450</td>
<td>0.258</td>
</tr>
<tr>
<td>l-Arabinose</td>
<td>0.045</td>
<td>0.354</td>
</tr>
<tr>
<td></td>
<td>0.225</td>
<td>0.301</td>
</tr>
<tr>
<td></td>
<td>0.450</td>
<td>0.250</td>
</tr>
<tr>
<td>d-Xylose</td>
<td>0.045</td>
<td>0.359</td>
</tr>
<tr>
<td></td>
<td>0.225</td>
<td>0.309</td>
</tr>
<tr>
<td></td>
<td>0.450</td>
<td>0.265</td>
</tr>
<tr>
<td>d-Mannitol</td>
<td>0.045</td>
<td>0.363</td>
</tr>
<tr>
<td></td>
<td>0.225</td>
<td>0.355</td>
</tr>
<tr>
<td></td>
<td>0.450</td>
<td>0.293</td>
</tr>
</tbody>
</table>

Varying amounts of phenolindo-2,6-dichlorophenol were then added and the tubes heated at 70° for 15 minutes. The tubes that were decolorized within that time were noted, and a further series of tubes made up, containing quantities of reagent distributed over a narrower range. The accuracy in this work appeared to be about 0.5 ml. of the phenolindo-2,6-dichlorophenol, although the originators of the method claimed to be able to distinguish differences to 0.01 ml. The results with this method are presented in Table I.

The influence of sugars and d-mannitol on the inhibition to heat coagula-
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tion was studied in the following manner. 1.5 ml. of an egg albumin solution containing 40.62 mg. of egg albumin per ml. were pipetted into test-tubes and 4 ml. of a phosphate buffer, pH 4.8, and 4.5 ml. of water, or of a solution containing the inhibiting agent, were added. The solutions were then heated at 70° for 15 minutes, filtered, and the nitrogen in a 2 cc. aliquot of the filtrate determined. The results with various sugars and mannitol are given in Table II. Each value given represents the average of at least quadruplicate coagulations and subsequent nitrogen determinations. In two additional series of experiments, sufficient d-glucose or d-fructose was added to saturate the solution of the egg albumin completely.

**Table II**

*Influence of Inhibiting Substances on Heat Coagulation of Egg Albumin*

The values given are increases in percentages, over controls, of non-coagulable nitrogen in the filtrate after removal of the coagulated protein.

<table>
<thead>
<tr>
<th>Substance</th>
<th>Concentration of inhibiting substance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.045 M</td>
</tr>
<tr>
<td>d-Glucose</td>
<td>3.2</td>
</tr>
<tr>
<td>d-Fructose</td>
<td>2.1</td>
</tr>
<tr>
<td>d-Mannose</td>
<td>2.2</td>
</tr>
<tr>
<td>L-Arabinose</td>
<td>7.7</td>
</tr>
<tr>
<td>d-Xylose</td>
<td>2.2</td>
</tr>
<tr>
<td>Sucrose</td>
<td>2.4</td>
</tr>
<tr>
<td>d-Mannitol</td>
<td></td>
</tr>
</tbody>
</table>

* About 4.6 M in respect to glucose.
† About 5.0 M in respect to fructose.
‡ About 0.8 M in respect to d-mannitol.

After the period of heating as described above, the solutions were dialyzed until no reducing sugar could be detected in the diffusate with Benedict's reagent. When the egg albumin so dialyzed was adjusted to pH 4.8, no coagulation occurred.

The first attempt to determine whether "symplexes," of a type similar to those described by von Przylecki and Cichocka, might be responsible for the inhibiting influence was made by comparing the amount of coagulation of the egg albumin first treated with sugar at pH 4.8, and subsequently heat-coagulated at that pH, with that first treated with sugar at a pH of 8.6, and subsequently heat-coagulated at a pH of 4.8. The sugars were added to the egg albumin at the pH indicated and the samples were coagulated at once or else allowed to stand for 96 hours at a temperature of about 5°. At the end of that period, these samples were also coagulated and the nitrogen determined in aliquot portions of each of the filtrates. Results are presented in Table III.
The second attempt to show "symplex" formation consisted of the coagulation of 5 ml. portions of a solution containing 60.5 mg. of egg albumin per ml. in the presence and in the absence of d-glucose. The coagulum in each case, after being washed, was partially hydrolyzed by being heated for 8 hours with 2 per cent hydrochloric acid in an oil bath at 120° and the hydrolysate was analyzed for reducing substances. Any unhydrolyzed protein was removed with lead acetate, the excess lead removed with potassium oxalate, and the reducing substances determined by the modified Benedict method for blood sugar (14). A photometer with a green filter of maximum absorption range of 5200 to 5800 Å. was used for comparison of the amounts of color developed. The standardization curve was made with d-glucose solutions containing 0.05 to 0.5 mg. per ml. The average of twelve determinations of the reducing substances, calculated as d-glucose in the partially hydrolyzed egg albumin, was 1.26 mg. with an average deviation from the mean of ±0.056, while the corresponding figure for the partially hydrolyzed egg albumin, coagulated in the presence of glucose, was 1.30 mg. with an average deviation from the mean of ±0.072.

**DISCUSSION**

The inhibition of sulfhydryl formation, as determined by two different methods, depends to some extent on the nature of the sugar used. The alcohol d-mannitol is less effective than the simple sugars. The amount of cysteine, as calculated from the sulfhydryl content, by the iodoacetic acid method, was 0.589 per cent; that by the phenolindo-2,6-dichlorophenol was 0.563 per cent. These values compare favorably with 0.56 to 0.61 per cent reported by Mirsky and Anson (15), 0.55 per cent reported by Rosner, 0.58 per cent reported by Kuhn and Desnuelle (16), 0.50 per cent reported by Greenstein (17), and that of 0.63 per cent given by Todrick and Walker (13). Based on the value 0.59 per cent, the percentage protection given by each of the different substances in Table I when used in 0.225 M concentration would be 17 per cent for d-glucose, 16 per cent for d-fructose, 14 per cent for d-mannose, 16 per cent for l-arabinose, 14 per cent for d-xylose, and

**TABLE III**

**Influence of Time of Contact, and pH, of Sugars on Coagulation of Egg Albumin**

The values given are for the percentage of nitrogen in the filtrate after coagulation based on the total nitrogen in the filtrate before filtration.

<table>
<thead>
<tr>
<th>Sugar, 0.3 M</th>
<th>Coagulated at once</th>
<th>Coagulated after 96 hrs. at 5°</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH 4.8</td>
<td>pH 8.6</td>
</tr>
<tr>
<td>d-Glucose</td>
<td>12.41</td>
<td>58.32</td>
</tr>
<tr>
<td>d-Fructose</td>
<td>12.53</td>
<td>46.98</td>
</tr>
</tbody>
</table>

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1 per cent for d-mannitol. These values are in most cases somewhat higher
than the values given in Table II for the influence of the same substances on
heat coagulation. The chief inference would be that some factor in addition
to the effect on the sulfhydryl group is involved in the subsequent coagula-
tion. Sørensen and Sørensen (18) a number of years ago showed that
denaturation did not produce an increase in soluble nitrogen, but subsequent
heating with water caused an increase in ammonia N and total soluble N.

That the inhibition is due to an influence on the native protein, and not
to a peptization of the coagulated protein, is indicated both by (1) the
influence on the sulfhydryl groups, and (2) the stabilization of egg albumin
when saturated with d-glucose or d-fructose. In the latter cases, no coagu-
lation occurred when practically all of the sugar was removed by dialysis,
and when the pH of the solution was adjusted to the isoelectric point of the
egg albumin. If the egg albumin had been previously denatured, coagu-
lation should have occurred (19).

The failure to show more inhibiting influence at higher pH does not
indicate the formation of “symplexes” as described by von Przylecki and
Cichocka, neither does the observation that, coagulated in the presence of
glucose, egg albumin has practically the same content of readily hydrolyz-
able reducing substances as of that coagulated in the absence of glucose.
These “symplexes,” as pointed out by von Przylecki and Cichocka, would
be very labile at a pH comparable to that occurring in a cell.

Work to be reported later will demonstrate that sugars decrease the
mobility of egg albumin. Preliminary work in connection with the Central
Brucella Station, Michigan State College (20), has shown that bovine
plasma saturated with glucose shows, after heating, a normal electrophoretic
pattern.

SUMMARY

d-Glucose, d-fructose, d-mannose, l-arabinose, d-xylose, and d-mannitol
inhibited the formation of sulfhydryl groups when egg albumin was heat-
denatured under specified conditions. These same substances, as well as
sucrose, increased the amount of non-coagulable nitrogen when egg albumin
was heat-coagulated under similar conditions. The inhibiting influence
toward heat coagulation does not increase with the increase of time of con-
tact of the agent with the egg albumin, even at a high pH. Egg albumin
coagulated in the presence of glucose does not contain significantly more
readily hydrolyzable reducing substances than does egg albumin coagulated
in the absence of glucose.

BIBLIOGRAPHY

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