DENATURATION CHANGES IN EGG ALBUMIN WITH UREA, RADIATION, AND HEAT

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The change produced in native proteins by various agents which results in loss of their characteristic properties and changes in solubility is called denaturation. The term, however, may be applied to structural and physical changes in the protein molecule which differ widely with the particular agent used.

Urea denaturation differs in many respects from both heat and radiation denaturation. Urea has a strong dispersive[1] action on proteins so that when a high concentration of urea is added to a protein the solution remains clear until the solution is diluted or dialyzed when a certain amount of insoluble protein is formed. Hopkins (1) found that the rate of denaturation of egg albumin by urea varied inversely with the temperature, but Diebold (2) found a positive temperature coefficient for the denaturation of fibrinogen by urea. Owing to its dispersive action high concentrations of urea will dissolve proteins coagulated by the action of heat or radiation, or other denaturing agents. Laporta (3) reports a certain amount of reversal of denaturation as a result of this dispersion by urea. Steinhardt (4) has shown that the functional properties of hemoglobin and pepsin are retained in urea solution so that the loss of solubility after treatment with urea may not be a true denaturation.

Many observers (2, 5, 6) have found that sulfhydryl groups appear after denaturation which are not detectable in native proteins. In the case of urea denaturation the number of SH groups is independent of the protein concentration and depends on the concentration of urea.

In the presence of urea most proteins split into smaller molecules. Burk and Greenberg (7) found that in 40 per cent urea hemoglobin had half of its normal molecular weight but that egg albumin was unchanged. Recently Williams and Watson (8) found some dissociation of egg albumin into smaller molecules in 50 per cent urea.

This investigation was undertaken with the idea of comparing the effect of different denaturation agents on molecular structure and shape and of studying the interrelations between the effects of different denaturation agents. In the course of the study certain new observations on urea denaturation were made.

[1] The term "dispersion" is used here to denote the opposite of "aggregation" as in the articles by Hopkins (1).

Methods

The material used was isoelectric egg albumin (0.9 per cent except where otherwise stated) prepared by the method given in previous publications (9, 10).

Ultraviolet radiation was given by the General Electric Portable A.C. Uviarc.

Measurements of the opalescence of the solution were made by means of the Tyndall meter and Macbeth illuminometer described in a previous publication (9) and in observations of the depolarization of the Tyndall beam a polarizing eye piece was used with the Macbeth illuminometer and readings taken with the nicol in two positions. The ratio of the dark to the bright component multiplied by 100 gave the per cent depolarization.

Optical rotation measurements were made with a Hilger polarimeter giving readings to 0.01°. The *D* line of sodium was used throughout and the experiments were carried out at room temperature.

Hydrogen ion determinations were made colorimetrically.

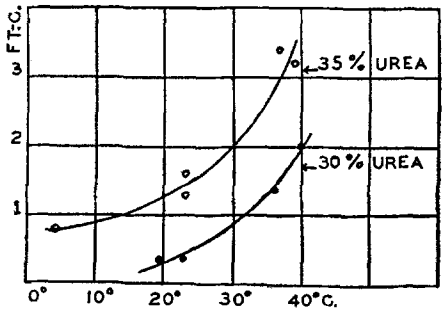

FIG. 1. Effect of temperature on rate of urea denaturation of egg albumin. Ordinates = opalescence of Tyndall beam in apparent foot-candles. Abscissae = temperature.

RESULTS

Urea Denaturation.—When urea is added to isoelectric egg albumin solutions in concentrations of 10 to 50 per cent there is a shift of pH to about pH 5.2–5.8 depending on the concentration of urea. Even if these solutions are brought to pH 4.8 they remain clear as long as the urea is present.

If the urea is dialyzed out and the solutions adjusted to the isoelectric point there is a precipitate formed and the degree of opalescence was taken as a measure of the denaturation. Contrary to the observations of Hopkins (1) the rate of denaturation was found to have a positive temperature coefficient, the rate increasing rapidly with temperature above 20° C. The results are given in Fig. 1 and Table I for solutions standing 2 hours at temperatures from 4–40° C.

The rate of denaturation at 40° C. for different concentrations of urea is shown in Fig. 2. The urea was added to isoelectric albumin bringing the pH to 5.4–5.6

and subsequently dialyzed and adjusted to pH 4.8. With concentrations of urea less than 20 per cent the rate of denaturation was too slow to be appreciable.

TABLE I

Temperature Coefficient of Denaturation of 0.9 Per Cent Egg Albumin by Urea

Temperature	Temperature coefficient	
	30 per cent urea	35 per cent urea
°C.		
10–20	—	1.33
20–30	2.0	1.66
30–40	2.5	2.0

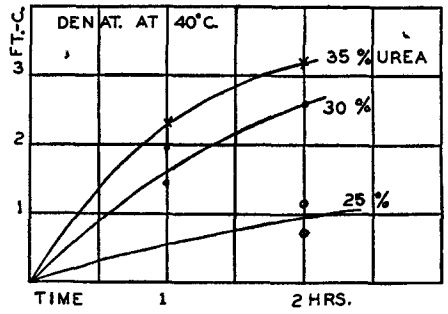

FIG. 2. Rate of urea denaturation of egg albumin at 40° C. Ordinates = opalescence of Tyndall beam in apparent foot-candles. Abscissae = time of immersion in water bath at 40° C.

TABLE II

pH	Urea concentration	Opalescence	
		6 days 21°	7 days 4°
		ft.-candles	*ft.-candles*
4.8	25	2.35	0.41
	30	3.70	1.38
	40	5.30	2.00

Although urea denaturation is very slow at 0° after 9 to 10 days in the ice box the following results are obtained (see Table II).

One may conclude, therefore, that 20 per cent urea produces practically no denaturation in a 0.9 per cent egg albumin solution. Urea denatures slowly at a concentration of 25 per cent. 35 per cent is rapidly effective at room temperature and 30 per cent is rapidly effective at a somewhat higher temperature.

Conflicting results on urea denaturation are undoubtedly due to the fact that the concentration necessary to produce denaturation varies with the protein used and its concentration as well as with the concentration of urea and the temperature of the solution.

Effect of Urea on Heat Denaturation.—It has been stated that urea, because of its dispersive action prevents the heat coagulation of proteins. It was found that in the presence of 50 per cent urea, egg albumin solutions brought to boiling will not precipitate until the urea is dialyzed out and the pH adjusted to 4.8. Also it was found that heat-coagulated albumin will redissolve in 50 per cent urea. However, the protein is dispersed without reversal of denaturation.

Concentrations of 20 to 40 per cent urea did not prevent the heat denaturation of albumin and indeed seemed to accelerate the denaturation. If tubes con-

TABLE III

Effect of Urea on Heat Denaturation

pH	Urea	Temperature		Opalescence
	per cent			*ft.-candles*
4.8	—	58° C.	½ min.	—
4.8	25	58° C.	½ min.	5.2
4.8	30	58° C.	½ min.	6.2
4.8	40	58° C.	½ min.	6.2
4.8	50	58° C.	½ min.	—
4.8	—	58–60° C.	10 min.	1.8
4.8	25	58–60° C.	10 min.	4.7
4.8	—	62° C.	3 min.	1.78
4.8	25	62° C.	3 min.	4.4

taining egg albumin only and egg albumin plus 25, 30, and 40 per cent urea, readjusted to pH 4.8, were heated simultaneously, opalescence appeared first in the tubes containing 30 and 40 per cent urea. These show some opalescence as the temperature of the water in which the tubes are immersed reaches 54° C. If the water is brought to 58° C. and the tubes then removed the tube containing egg albumin is still clear and those containing urea show a dense precipitate, the precipitate being heavier in the tubes containing 30 and 40 per cent than in the tube containing 25 per cent.

Some results are summarized in Table III.

This would seem to indicate that urea in concentrations too low to split the molecule and disperse the heat-denatured protein lowers the heat coagulation temperature of egg albumin and accelerates the rate of heat coagulation. Actually a different interpretation was found to be more consistent with the facts as it is probable that heat below the temperature of heat coagulation breaks the urea-protein complex and allows the precipitation of urea-denatured protein.

Effect of Urea on Radiation Denaturation.—In a previous publication (9) it was stated that egg albumin exposed to ultraviolet radiation at 4° C. remained clear until heated for a short time to a moderate temperature (40° C.). With the stronger arc used in these experiments, and consequently less perfect temperature control, there was slight opalescence after radiation on ice, although this opalescence was prevented by adding 25 to 30 per cent urea to the solutions. The zone in which opalescence occurs is also narrowed in the presence of urea.

If the solutions are radiated on ice and then put in a water bath at 40° C. the opalescence that normally occurs is diminished by 25 per cent urea and completely prevented by 35 per cent (see Fig. 3).

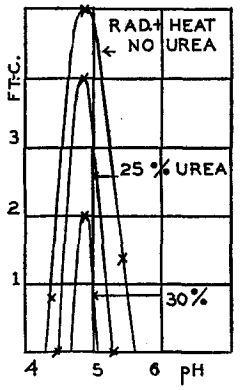

FIG. 3

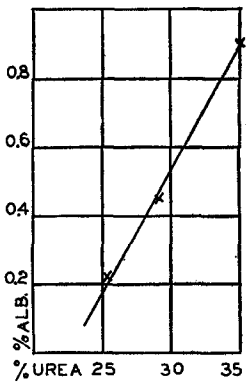

FIG. 4

FIG. 3. Effect of urea on denaturation by radiation followed by heating to 40° C. Ordinates = opalescence of Tyndall beam in apparent foot-candles. Abscissae = pH.

FIG. 4. Concentration of urea necessary to prevent aggregation in egg albumin after radiation and heat (40° C.) at the isoelectric point. Ordinates = concentration of albumin in per cent. Abscissae = concentration of urea in per cent.

That radiation changes have taken place is evidenced by color and smell and by the fact that the solutions precipitate when the urea is dialyzed out. As 30 to 35 per cent urea will denature fairly rapidly at 40° there will be urea denaturation as well as radiation denaturation, but as the results are the same if the urea is dialyzed out before heating to 40° C. it is probable that the radiation changes take place in the presence of urea and urea only prevents the flocculation of the denatured protein. If the concentration of egg albumin is reduced the opalescence is abolished by a lower concentration of urea. A 0.45 per cent albumin solution remained clear if radiated and heated in 30 per cent urea. The concentration of urea needed to prevent flocculation after radiation and moderate heat is proportional to the concentration of the protein (see Fig. 4).

If albumin solutions are radiated at pH 7.4 or 3.8 and heated to 40° C. for an hour they remain clear but precipitate on being brought to pH 4.8. This pre-

cipitation is prevented if 25 per cent urea is present but the solutions precipitate if the urea is dialyzed out.

Chick and Martin (11) found heat denaturation to be a reaction between protein and water with an extraordinarily high temperature coefficient. Heat coagulation involves two processes, the denaturation of the protein and the separation of the denatured protein in flocculated form.

The coagulation of proteins after exposure to ultraviolet radiation has been shown (9) to take place in three steps. Step 1 is a change produced by radiation shorter than λ 310 mμ which takes place at any temperature and any pH. Step 2 is a heat change taking place in the light-denatured molecule with a high temperature coefficient which takes place at any pH and is effective at a temperature which does not produce any appreciable heat denaturation in the unradiated protein. Step 3 is the aggregation of the light- and heat-denatured molecules which occurs only near the isoelectric point.

One may summarize these changes as follows:

Denaturing agent	Steps in process of denaturation		
	(*a*)	(*b*)	(*c*)
Heat	—	Heat denaturation	Separation of denatured protein in flocculated form
	(*a*)	(*b*)	(*c*)
Ultraviolet radiation followed by moderate heat	Light denaturation	Heat change in light-denatured molecule	Separation in flocculated form

Urea in concentrations below 50 per cent (the concentration at which it splits the egg albumin molecule) appears to accelerate change (*b*) in heat denaturation although the precipitate formed is probably due to urea-denatured not to heat-denatured molecules. Above 50 per cent it prevents change (*c*) as the result of splitting the molecule.

In the case of radiation denaturation, steps (*a*) and (*b*) take place when egg albumin solutions are radiated and subsequently heated in the presence of urea. The third step, (*c*), of flocculation is prevented by 35 per cent urea if the solutions are radiated and heated at pH 4.8 and by 25 per cent urea if they are radiated outside the zone of opalescence. This is a concentration of urea which does not prevent the flocculation accompanying heat denaturation. As the concentration of urea needed to prevent step (*c*) in radiation denaturation is proportional to the concentration of protein, there is obviously a urea-protein complex formed which reacts to radiation and to heat in a way that differs from the protein molecule alone. The changes may be diagrammed as in Fig. 5. Al-

though for diagrammatic purposes the complex is shown as one molecule of protein plus one molecule of urea the relative concentration would suggest that the protein molecule may be enveloped in a shell of urea molecules. According to this interpretation the urea-protein complex will not aggregate producing flocculation, hence preventing step (*c*) in radiation denaturation, as the temperature of 40° C. used in radiation denaturation is not high enough to break down the complex. In heat denaturation, however, a temperature of 54° and over breaks down the urea-protein complex before a heat change *per se* takes place in the molecule and there is consequent flocculation of the urea-denatured molecule. When the molecules have been split by urea in concentrations of 50 per

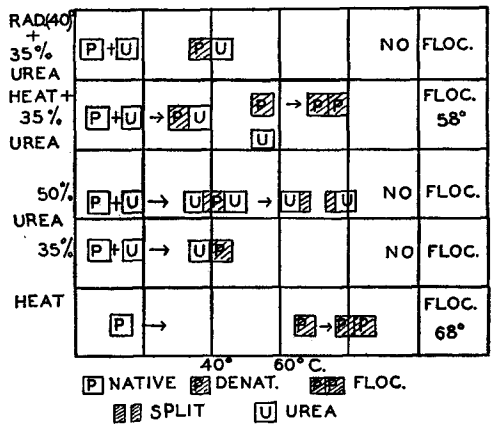

FIG. 5. Diagram showing interaction of heat, radiation, and urea on egg albumin molecule at different temperatures and concentrations of urea.

cent or over they are apparently no longer capable of flocculation until the urea is removed by dialysis.

Tyndall Beam Results.—An attempt was made to investigate the effect of ultraviolet radiation, heat, and urea on the shape of the albumin molecule by the depolarization of the Tyndall beam. The Tyndall beam from a solution in which the molecules are roughly spherical and small compared with the wave length of light is completely polarized. Depolarization is caused by increase in asymmetry or increase in size of the particles. The results were not satisfactory as the increase in size when opalescence developed at the isoelectric point completely masked the effect of change in shape. Opalescence less than 1.5 foot-candles was found to be determined more by asymmetry than by change in size. The results from the clear and slightly opalescent solutions are of doubtful significance, but are given in Table IV and indicate the following results.

1. The egg albumin molecule after radiation only has a fair degree of sym-

metry except at the isoelectric point and the same is true after radiation and heating to 40° C.

2. Asymmetry develops with aggregation which is prevented in the presence of urea.

3. Even when there is some degree of aggregation as evidenced by opalescence the presence of urea prevented any great degree of depolarization so that the protein-urea complex tends to remain a fairly symmetrical molecule.

TABLE IV

Depolarization of Tyndall Beam

Treatment	pH	Urea	Total opalescence	Bright component	Dark component	Depolarization
		per cent				*per cent*
Native albumin	4.8	—	0.07	0.13	0.05	37
Urea-denatured	4.8	35	0.2	0.4	0.10	25
	4.8	35	1.3	1.9	0.5	26
Radiation only	4.4	—	0.85	1.33	0.29	22
	4.8	—	0.14	0.245	0.1	40
	5.4	—	0.5	0.85	0.18	21
Radiation + urea	4.8	30	0.14	0.36	0.09	25
Radiation + heat 40° + urea	4.6	25	0.35	0.6	0.12	20
	5.0	25	1.0	1.7	0.34	20
	5.2	25	0.57	1.05	0.2	20
Heat 100° at pH 3.8 or 7.4 brought to different pH after heating	4.4	—	0.82	1.35	0.28	20
	4.8	—	1.75	2.5	1.0	40
	5.8	—	0.92	1.6	0.34	21
	5.0	25	2.2	3.4	1.28	37
	5.6	25	0.55	0.95	0.17	18

4. Heat denaturation at a pH outside the zone of opalescence leaves molecules fairly symmetrical until they are brought near the isoelectric point.

5. Urea does not prevent this asymmetry in heat-denatured molecules near the isoelectric point in 25 per cent concentration, which is probably due to the fact that heat breaks down the protein-urea complex.

These results tend to confirm the picture of the reaction of the protein molecule and of the protein-urea complex to radiation and heat that is given in Fig. 5.

Optical Rotation.—The change in optical rotation with heat denaturation previously observed (12) was confirmed and additional results obtained with

ultraviolet radiation and urea denaturation. Observations on undenatured albumin were made over a range of concentration and from pH 3.4 to 10.5. The results are given in Table V.

TABLE V

Optical Rotation of Egg Albumin. Effect of Denaturation and Changes in pH and Concentration

Condition	Concentration	pH	(Angle of rotation)	$[\alpha]_D^{22}$	Denatured
	per cent			*deg./dm.*	*per cent*
Fresh	1.2	4.8	−0.35°	−29.0	
	0.6	4.8	−0.34°	−28.0	
	0.3	4.8	−0.19°	−32.0	
	0.15	4.8	−0.10°	−33.0	
Fresh	1.2	3.4	−0.34°	−28.0	
		4.8	−0.34°	−28.0	
		6.0	−0.34°	−28.0	
		6.8	−0.33°	−27.5	
		7.4	−0.32°	−27.0	
		9.0	−0.33°	−27.5	
		10.5	−0.34°	−28.0	
Boiled 5 min.	1.2	3.4	−0.60°	−56.0	
		6.4	−0.74°	−61.7	100
		7.2	−0.61°	−50.8	
	0.6	7.0	−0.60°	−50.0	
Ultraviolet radiation					
20 min. (4° C.)	1.2	6.4	−0.39°	−32.5	35
1 hr. (4° C.)	1.2	6.4	−0.40°	−40.0	50
20 min. (4° C.)	0.6	7.0	−0.38°	−32.0	
40 min. (4° C.)	0.6	7.0	−0.50°	−42.0	
Urea 25 per cent	0.6	4.8	−0.33°	−27.5	
45 per cent	0.6	4.8	−0.34°	−28.0	
60 per cent	0.6	4.8	−0.43°	−36.0	

As has been previously observed (13) it was found that fresh undenatured albumin shows no variation in optical rotation between pH 3.4 and 10.5. There was evidence of a slight increase in optical rotation in a very dilute solution (0.15 per cent) but observations with such dilute solution are subject to large errors and the increase observed may not be significant. Boiling for 5 minutes at pH 3.4 or 6.4 → 7.2 approximately doubled the optical rotation, the increase being greater, as pointed out by Barker (12) the closer the pH is to the isoelectric point.

When albumin is exposed to ultraviolet radiation at a temperature of 4° C. or

less, the first step in the denaturation process occurs, the coagulation which results from radiation being the result of a three-step process (9). The result of the first step is to increase the optical rotation and the increase is roughly proportional to the amount denatured as determined gravimetrically by subsequent coagulation of the light-denatured protein. Indeed a measure of the optical rotation of the radiated protein would serve as an excellent and very simple method for estimating the amount of denaturation following radiation for this particular protein, and shows a similarity here between radiation and heat denaturation.

Urea concentrations up to 45 per cent had no effect on optical rotation but there was a marked increase at 60 per cent urea. At higher concentrations it was impossible to obtain readings.

Where changes in optical rotation can be checked with ultracentrifuge determinations of molecular weight it is evident that association of molecules into larger complexes is accompanied by a decrease in optical rotation and dissociation by an increase. The stable values of optical rotation in all proteins in the pH range where the molecular size of proteins remains constant and the increase in optical rotation outside this range is one example of this. Association and dissociation, however, are not the only possible causes of change in the optical rotation values of a protein molecule.

Egg albumin denatured with 40 per cent urea has the normal molecular weight of 35,000 to 40,000 but it has been reported (8) that there is some dissociation of the molecule with concentrations of urea above 50 per cent. The change in optical rotation with urea above 50 per cent is probably accounted for by dissociation of the molecule.

The increase in optical rotation with heat and radiation denaturation, however, must be due to structural changes and not to change in size. No ultracentrifuge results for the molecular weight of egg albumin denatured outside the pH range where aggregation occurs are available. However, osmotic pressure measurements show no evidence of dissociation to account for the observed optical rotation changes.

One may conclude, therefore, that the egg albumin molecule shows an increase in optical rotation as the result of structural changes when denatured by radiation or heat. Urea denaturation does not affect the optical rotation of egg albumin, but splitting of the molecule by high concentrations of urea increases the optical rotation.

CONCLUSIONS

The extent of urea denaturation depends on the concentration of protein and urea and also on the temperature of the solution. Egg albumin solutions (0.9 per cent) are not denatured by 20 per cent urea, denature slowly with 25 per

cent urea, and denature rapidly with 35 per cent urea at room temperature. At a higher temperature 30 per cent urea is rapidly effective.

Denaturation of the egg albumin molecule by radiation or by heat is accompanied by structural changes as evidenced by optical rotation values, but is not accompanied by association or dissociation of the molecule in the pH range outside the zone in which aggregation follows denaturation.

Denaturation of the egg albumin molecule by urea produces no change in optical rotation until the concentration of urea is high enough to dissociate the molecule.

In the presence of urea a urea-protein complex is formed in which the protein is denatured but cannot flocculate because of the dispersive action of the urea. This prevents flocculation of proteins exposed to radiation and subsequent heating to 40° C. as the urea-protein complex is not broken down at a temperature of 40° C. The presence of urea therefore prevents the flocculation of proteins denatured by radiation.

The urea-protein complex is broken down by heating to 55–58° C. so that the molecules aggregate at a temperature below the temperature of rapid heat denaturation. This appears to be an acceleration of heat denaturation or a lowering of the heat denaturation temperature, but in reality is an effect of heat on the urea-protein complex which frees the urea-denatured protein and permits its aggregation.

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