THE TEMPERATURE COEFFICIENT OF THE UREA DENATURATION OF EGG ALBUMIN

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When urea acts on a protein the progress of the reaction depends upon the concentration of protein, the concentration of urea, the pH, and the temperature of the solution. In the change spoken of as urea denaturation, there are at least four reactions involved: (1) the formation of a urea-protein complex; (2) the denaturation of the molecule with change in its typical biological properties; (3) the change in solubility of the molecule, which is often spoken of as denaturation; (4) the splitting of the molecule (at high concentrations of urea only).

Unfortunately, it is not easy or even possible to separate these four reactions. Although the swelling of proteins in urea solutions, freezing point determinations of proteins in urea, and x-ray patterns have given definite evidence of the existence of a urea-protein complex, there is no work so far reported that measures the rate of reaction 1, and it is generally assumed that it takes place so rapidly, especially at high concentrations of urea, that it does not affect the rate of the other three reactions. It is a preliminary reaction and if not followed by subsequent changes in the protein molecule, is probably reversible. It may, however, be a factor in the slow rate of denaturation of egg albumin with urea concentrations under 20 per cent (1) and may determine the critical concentration below which urea produces no inactivation in viruses (2).

In the case of hemoglobin, it has been shown (3) that reaction 4 can proceed without any effect on the specific biological properties of the molecule; *i.e.*, without reaction 2 taking place.

In the case of the effect of urea on tobacco mosaic virus, which has been exhaustively studied by Lauffer and Stanley (4–6), the methods used were measurements of the rate of reaction 4, the splitting of the molecule. Only one method in which the urea-protein mixture was diluted so as to precipitate the urea-denatured virus measured reaction 3, the rate of change of solubility of the protein. As Lauffer finds that the rate of change of solubility runs parallel with the rate of splitting of the molecule, except in the first few minutes of the reaction, he concludes that the split protein is the protein which shows loss of solubility in dilute solution. He does, however, state that the virus may lose its infectivity before the protein disintegrates. In other words, reaction 2 may precede reactions 3 and 4.

The picture in the case of egg albumin is very different. There is no method for measuring reactions 1 and 2, and the progress of denaturation is usually followed by measuring the rate of reaction 3, the loss of solubility in dilute solutions. This proceeds at urea concentrations too low to split the molecule (1). When the concentration of urea is high enough to split the molecule, it is found in the experiments reported here that the split molecule is soluble after the urea has been dialyzed out. Consequently, at high concentrations of urea where both reaction 3 and reaction 4 take place, the rate of formation of insoluble protein, which is the usual measurement of denaturation, is decreased in proportion to the rate of progress of reaction 4, the splitting of the protein.

In comparing denaturation changes in different proteins, it is important to analyze the reactions involved and compare results on the same reactions. In the case of tobacco mosaic virus, the split denatured protein is insoluble in dilute solutions so that measurements of reactions 3 and 4 run parallel. In the case of egg albumin, although denaturation makes the molecule insoluble in dilute solutions, splitting of the molecule makes the denatured egg albumin more soluble, so that the faster reaction 4 proceeds the less apparent denaturation there is, if denaturation is measured by the amount of insoluble material formed. It is not possible to tell whether reaction 3 must precede reaction 4, or whether the two reactions proceed independently and simultaneously. The results reported in this paper tend to support the view that denaturation (reaction 3) precedes splitting of the molecule (reaction 4).

Hopkins (7) studied the temperature coefficient of the denaturation of 5 per cent egg albumin solutions at pH 6.0 in the presence of 60 per cent urea. He found that denaturation, as measured by the insoluble protein formed on dilution, progressed more rapidly at 0° than at room temperature. Lauffer (6), studying the rate of splitting of tobacco mosaic virus with 6 M urea found that the specific reaction velocity was a minimum at room temperature and increased at higher or lower temperatures. He suggests that this U-shaped temperature coefficient curve may be characteristic of the denaturation of proteins, and that with changes in the concentration of urea the bottom of the U may shift, giving a positive temperature coefficient in the 0–40°C. range for low concentrations of urea, and a negative temperature coefficient at high concentrations. This U-shaped curve, he believes, is due to the fact that there are several simultaneous reactions going on, some with a positive and one at least with a negative temperature coefficient.

In the case of egg albumin, where splitting makes the denatured protein soluble, the results of the experiments reported here with different concentrations of urea show that the negative temperature coefficient reported by Hopkins is probably due to the fact that both reactions, denaturation (reaction 3) and splitting (reaction 4), have positive temperature coefficients. If reaction 4 has a higher temperature coefficient than reaction 3, it will decrease the

amount of insoluble protein formed as the temperature increases and therefore it will decrease the apparent rate of denaturation. This is actually the interpretation given his results by Hopkins who says that the figures present the "simulacrum" of a negative coefficient, and that he believes this to be due to the fact that "dissociation increases with increase in temperature sufficiently to account for the observed diminution in rate of denaturation." This is very different from saying that the urea denaturation of egg albumin has a negative temperature coefficient, which has been the usual interpretation given his results by other investigators. During the period of observation (3 hours) he found no diminution in the amount of insoluble protein, but he states also that if the urea-protein mixtures stand several days they remain clear on dialysis, which is obviously due to the splitting of all the denatured protein, and an apparent reversal of the denaturation process when that is measured by the amount of insoluble protein formed.

The following experiments show clearly that both denaturation (reaction 3) and splitting (reaction 4) have positive temperature coefficients, but at high concentrations of urea, where splitting becomes rapid, the increase in rate of splitting with increase in temperature will result in an apparent diminution in the rate of denaturation, when this is measured by the amount of insoluble protein formed on dilution or dialysis. Consequently, experiments performed with low concentrations of urea (less than 35 per cent) will show a positive temperature coefficient (1), as the amount of splitting has not been great enough to decrease the amount of insoluble protein formed. And experiments performed with 50 to 60 per cent urea, where splitting takes place rapidly, will give the appearance of a negative temperature coefficient (7).

Method

The material used was isoelectric egg albumin (0.6 per cent before addition of urea) prepared by the method given in previous publications (8, 9). On addition of urea there was a shift in the pH value to pH 5.4.

Measurements of the opalescence of the solution were made by means of the Tyndall meter and Macbeth illuminometer, as previously described (9). The opalescence of the Tyndall beam, which serves as a measure of the amount of insoluble material present, is given in apparent foot-candles.

Results are given for egg albumin solutions to which urea had been added in concentrations of 40 and 50 per cent. These urea-albumin solutions were kept at 13°, 25°, and 40°C., for varying periods of time. They were then dialyzed against distilled water to remove the urea. This resulted in precipitation of the egg albumin molecules, in which reaction 3 had taken place, and in which reaction 4 had not taken place. The amount of insoluble material was measured by the opalescence of the Tyndall beam and is a measure of the amount of reaction 3, minus the amount of reaction 4 that had taken place.

RESULTS

Denaturation with 50 Per Cent Urea.—In Table I the results found with 50 per cent urea are given for solutions kept at 13°, 25°, and 40°C. for varying periods of time. These figures are average values found from a series of observations at each temperature and give the amount of insoluble material present as determined by the opalescence of the Tyndall beam in apparent foot-candles. Part of these results are shown also in Fig. 1.

From the results given in Table I and Fig. 1, it is obvious that any calculation of temperature coefficients from the amount of insoluble material formed is meaningless when the urea concentration is high enough to result in rapid splitting of the denatured molecule, as the opalescence is the result of two antagonistic reactions both with a positive temperature coefficient. As the temperature increases from 13–40°C. the highest level of opalescence reached becomes lower and the time required to reach this highest level becomes shorter. The rate of splitting of the molecule is apparently slow at 13°C. with 50 per cent urea as, after 69 hours in the ice box at this temperature the opalescence in the solutions after dialysis is almost as high as in solutions kept at room temperature for 12 hours, and higher than those kept at room temperature for 24 hours.

TABLE I

Time	Amount of insoluble protein measured by Tyndall beam readings in apparent foot-candles		
	40°C.	25°C.	13°C.
10 min.	2.7		
15 "	3.0	3.5	
20 "	2.7		
30 "	2.2	4.6	3.7
45 "	2.1		
50 "	1.83		
1 hr.		5.5	5.2
1.5 "	1.35	5.3	6.0
2 "	1.17	4.8	5.4
2.5 "			5.2
3 "		4.5	4.8
5 "			3.6
11 "		3.2	
24 "		2.25	3.3
55 "		1.82	
69 "			2.75

The rate of splitting must increase rapidly with temperature between 25 and 40°C., as at 40°C. the degree of opalescence begins to decrease long before it reaches a high value, and the maximum opalescence reached is only 3 foot-candles compared with 6 for solutions kept at 13°C.

These results seem to differ from those of Hopkins (7), where the amount of insoluble protein was still increasing after 3 hours at 37°C. But in his experiments he was using a 5 per cent albumin solution, and in such a solution the equilibrium between the two reactions (denaturation and splitting) would be different from that found in the much more dilute albumin solutions used in these experiments.

One may conclude, therefore, that when denaturation of egg albumin is measured by the amount of insoluble protein formed with 50 per cent urea, the

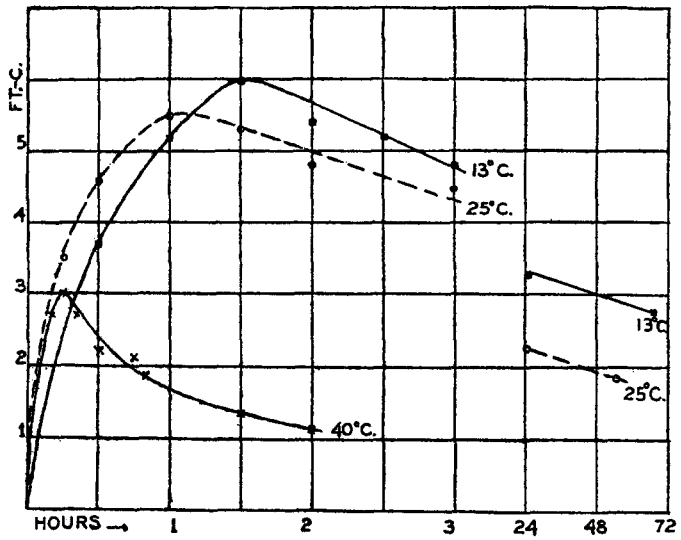

FIG. 1. Denaturation of egg albumin in 50 per cent urea at 13°, 25°, and 40°C. Ordinates = opalescence Tyndall beam in apparent foot-candles (a measure of the amount of insoluble protein present). Abscissae = time in hours.

results observed are due to two reactions, one increasing the amount of insoluble material (reaction 3), and the other decreasing it (reaction 4). As a result, the actual value of the temperature coefficient cannot be calculated, but it is obvious that both reactions have a positive temperature coefficient and that reaction 4 probably has a higher positive temperature coefficient than reaction 3, between 25 and 40°C.

Denaturation with 40 Per Cent Urea.—In Table II and Fig. 2 results are given for solutions containing 40 per cent urea, kept for varying periods of time at 13°, 25°, and 40°C. The figures are average values from a number of observations.

At this concentration the splitting of the molecule (reaction 4) is slower and reaction 3 proceeds fast enough so that at 40°C. the opalescence reaches its

maximum level, before any effect of splitting becomes apparent. The curves for the rate of development of opalescence at 13 and 25°C. are almost the same although the material at 25° reaches a maximum opalescence somewhat sooner. The fact that reaction 4 is progressing more rapidly at 25° is evident after the

TABLE II

Time	Amount of insoluble protein measured by Tyndall beam readings in apparent foot-candles		
	40°C.	25°C.	13°C.
15 min.	5.7		
30 "	6.4	2.75	
45 "	6.6		3.3
1 hr.	6.7	3.9	
1.5 "	6.2	4.6	4.3
2 "		5.4	5.1
3 "		6.0	
4 "		6.0	6.2
5 "		5.5	5.7
8 "		4.15	5.48
24 "		1.92	
48 "		1.15	4.4
72 "		0.72	3.3

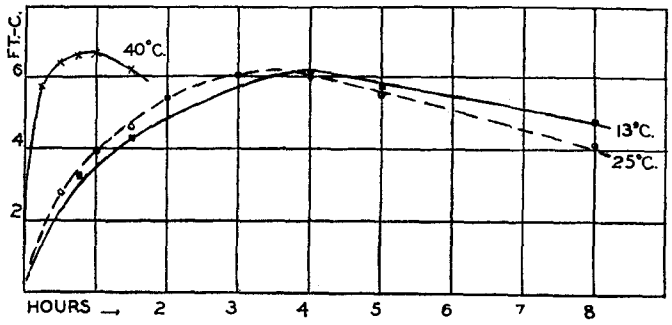

FIG. 2. Denaturation of egg albumin in 40 per cent urea at 13°, 25°, and 40°C. Ordinates = opalescence of Tyndall beam in apparent foot-candles (a measure of the amount of insoluble protein present). Abscissae = time in hours.

material has stood for a day or more. By that time the degree of opalescence in the material at room temperature has decreased very markedly while it is still high, though less than its maximum value, in the material at 13°C. The fact that the decrease in opalescence at 25°C. after several days is slightly greater in 40 per cent than in 50 per cent urea is probably not significant. The variations in room temperature over a period of several days would probably

be sufficient to account for this inconsistency, and actually fewer observations were made on material standing for several days, so that the 48 to 70 hour values are less accurate than those made up to 5 hours.

An additional fact of interest is this. If material which has stood for several days at either 13 or 25°C., until a large proportion of the molecules have been split, is then dialyzed and filtered until it is absolutely clear of suspended material, the filtrate will not precipitate on boiling. In other words, the changes produced by splitting are such that the change usually spoken of as denaturation (*i.e.* change in solubility) can no longer take place in the split molecules.

CONCLUSION

Evidence is brought forward to show that at concentrations of urea high enough to split the egg albumin molecule the solubility changes produced by urea are profoundly modified. The degree of precipitation after dialysis is the net result of two changes produced by the urea: the first, normally spoken of as denaturation, which makes the protein insoluble in dilute solution and the second, a splitting of the molecule which makes it soluble. These two reactions may proceed independently and simultaneously or the second reaction may follow the first, taking place in the denatured molecule only. In view of the decrease in the opalescence with time, the latter process is more probable.

Both of these reactions have positive temperature coefficients, but as the concentration of urea increases the second reaction is more affected by increase in temperature than the first, and consequently the resulting opalescence decreases rather than increases with temperature. This accounts for and explains reports of negative temperature coefficients of denaturation, when denaturation is measured by the amount of insoluble material found on dilution.

The occurrence of these two reactions, one leading to an increase and the other to a decrease in the amount of insoluble protein, should be taken into account when denaturation changes in egg albumin with urea are studied.

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