IV. THE DENATURATION OF PROTEINS. PART V.

DENATURATION BY ACID.

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IN earlier work carried out in this laboratory by P. S. Lewis [1926, 1927] on the heat denaturation of oxyhaemoglobin and egg-albumin it was shown that the unimolecular velocity constant characterising the process had a minimum value at the neutrality point of water (namely $p_H = 6.76$ at 37°), and, further, that the process appeared to be catalysed approximately to the same extent by hydrogen ions and by hydroxyl ions. In the region of minimal velocity it was also found that the denaturation possesses an extremely high critical increment, namely of the order 77,000 cal. for oxyhaemoglobin, and 130,000 cal. for albumin. The temperature range for which these values were obtained was in round numbers 60° to 70° .

In view of these facts it was considered to be of importance to extend the p_H range as far as possible in the direction of increasing acidity, such extension necessarily involving the employment of lower ranges of temperature in order to deal with reaction velocities which would not be immeasurably rapid.

The present communication contains a summary of the results obtained. The proteins investigated, namely oxyhaemoglobin and albumin, were prepared and purified by the same methods as those employed by Lewis [1926, 1927]. Lewis's methods of determining the extent of denaturation at the various time intervals were also followed, with the exception that for denaturation at relatively low temperatures, such as 18° , it was necessary not only to cool the sample withdrawn, but also to adjust its p_H by addition of alkali to that of the isoelectric point to ensure that the denaturation itself is stopped, and also that all the protein denatured is precipitated.

The velocity of denaturation has been determined at 18° , 25° , 37° and 45° , over different ranges of p_H . The velocity constants given in the tables are in all cases calculated from the ordinary unimolecular expression in which the logarithm is to the base e and time is expressed in seconds. The stock solutions contained 0.5 % ammonium sulphate. It is known that this concentration of the salt does not appreciably affect the velocity of denaturation, and at the same time the ammonium sulphate not only acts as a preservative, but also ensures that the flocculation of the denatured protein shall be very rapid compared with the denaturation process itself.

All the results quoted are the mean values of concordant experiments. For the case of oxyhaemoglobin the average divergence of the mean values does not exceed 2 to 3 %; for albumin the error is somewhat larger. As a rule, the denaturation was followed to the extent of over 50 % in any individual run. The p_H was determined by means of the quinhydrone electrode at 25° in all cases.

Acid denaturation of oxyhaemoglobin.

Temperature 18°		Temperature 25°	
p_H of solution	$k_{uni} \times 10^4$	p_H of solution	$k_{uni} \times 10^4$
4.08	10.95	4.08	17.61
4.13	8.19	4.13	12.49
4.30	4.58	4.32	5.25
4.39	2.76	4.39	4.30
4.40	2.71	4.41	3.73
4.49	1.69	4.52	2.29
4.56	1.07	4.64	1.27
—	—	4.77	0.51

Temperature 37°		Temperature 45°	
p_H of solution	$k_{uni} \times 10^4$	p_H of solution	$k_{uni} \times 10^4$
4.11	30.65	—	—
4.15	22.71	4.23	25.30
4.31	12.26	4.45	11.10
4.48	6.31	4.52	7.26
4.51	5.15	4.63	4.36
4.60	3.22	4.74	2.74
4.68	2.19	4.90	1.21
4.85	0.80	5.10	0.59
		5.50	0.096

Acid denaturation of egg-albumin.

In connection with this protein previous workers have observed a change in p_H during the course of heat denaturation. Consequently in the present series of measurements several determinations of p_H values were made by means of the quinhydrone electrode at various points during the course of a run, and it can be quite definitely stated that, over the temperature range and p_H range investigated by the writer, there appeared to be no sensible alteration in p_H as a consequence of denaturation.

Temperature 25°		Temperature 37°	
p_H of solution	$k_{uni} \times 10^5$	p_H of solution	$k_{uni} \times 10^5$
1.02	1.59	1.02	30.0
1.17	1.35	1.32	8.83
1.35	0.82	1.46	7.00
1.52	0.64	1.58	5.56
1.72	0.48	1.62	5.68
		1.80	3.57
		1.99	2.16
		2.25	1.08
		2.55	0.91
		3.83	0.06

Temperature 45°			
p_H of solution	$k_{uni} \times 10^4$	p_H of solution	$k_{uni} \times 10^4$
1.34	3.88	2.18	0.96
1.50	2.84	2.30	0.82
1.66	2.45	2.39	0.65
1.96	1.61	2.68	0.35
1.98	1.73	3.52	0.12

The critical increment.

This quantity, E , which represents the energy of activation of the denaturation process is calculated from the usual expression

$$\log \frac{k_2}{k_1} = \frac{E}{R} \left(\frac{1}{T_1} - \frac{1}{T_2} \right)$$

where k_2 and k_1 are the observed velocity constants at the two temperatures T_2 and T_1 . The values of E corresponding to the temperature range and to a number of p_H values within the limits quoted above are recorded in the following tables. It may be pointed out that errors in the individual velocity constants become magnified in the calculation of the E values therefrom. The values quoted are consequently rounded.

Critical increment of the acid denaturation of oxyhaemoglobin.

	Temp. range 18°-25°	Temp. range 25°-37°	Temp. range 37°-45°
p_H	E in cal.	E in cal.	E in cal.
4.1	11,900	—	—
4.2	8,600	11,100	—
4.3	8,300	14,100	(6,600)
4.4	9,100	12,800	(4,000)
4.5	11,300	11,700	12,000
4.6	—	9,100	12,000
4.7	—	12,400	13,900
4.8	—	—	12,500

Critical increment of the acid denaturation of egg-albumin.

	Temp. range 25°-37°		Temp. range 37°-45°
p_H	E in cal.	p_H	E in cal.
1.1	36,200	1.4	36,200
1.2	35,900	1.5	37,000
1.3	35,800	1.6	39,000
1.4	35,300	1.7	40,000
1.5	35,400	1.8	40,000
1.6	36,400	1.9	43,100
1.7	36,800	2.0	45,300
		2.1	48,100
		2.2	49,000
		2.3	48,200

DISCUSSION.

Even when full allowance is made for inevitable experimental error the striking result, shown in the foregoing tables, is that in the region of moderate acidity the critical increment of denaturation, both for oxyhaemoglobin and for egg-albumin, is very much lower than it is in the neighbourhood of the

neutrality point of water in which region the process is known as heat denaturation. It is not proposed in the present paper to attempt to offer any explanation of this. It is not even known with certainty, as yet, whether or not the moderate values of E which hold for the low p_H range and relatively low temperature tend to merge gradually into the extremely high values of E obtained at the high p_H range and relatively high temperature characteristic of heat denaturation. If the change is a gradual one this would constitute an argument of some weight pointing to the fundamental identity of acid denaturation and heat denaturation respectively. Further work is being undertaken in this laboratory in this connection.

As we have just had occasion to raise the question of the relation of heat denaturation to denaturation by acid, it may not be out of place to draw attention to yet another mode whereby denaturation is said to be brought about, namely, mechanical denaturation. In this connection certain results obtained by Wu and Ling [1927] may be cited to the effect that, in contrast to other forms of coagulation which involve denaturation as a distinct stage, mechanical treatment (shaking) leads directly to the observation that maximum velocity occurs at the isoelectric point. Under ordinary conditions the speed of denaturation is slow compared with that of the (subsequent) flocculation of denatured material. As a consequence of mechanical treatment it would seem that the denaturation process has been greatly speeded up, so that the total observed effect is essentially a coagulation rate which would naturally be a maximum at the isoelectric point. So far as this evidence goes it affords some slight support to the view that denaturation is essentially a distortion or opening up of the protein unit, usually brought about as a consequence of localised chemical changes in the unit, but possibly much more rapidly brought about by mechanical treatment.

THE INFLUENCE OF FORMALDEHYDE UPON THE FLOCCULATION OF DENATURED PROTEIN.

Flocculation of a protein is the process which is subsequent to denaturation and is rendered possible by it. Flocculation is usually ascribed to the interaction of amino- and carboxyl groups situated on contiguous colloid units as a consequence of which these units increase in size by aggregation and eventually are coagulated. If this view be correct, then, if by any means either of these groups be blocked or inactivated, the resulting protein should not exhibit flocculation. To this end formaldehyde at various concentrations was caused to react with protein and the resulting material examined at various p_H values including the isoelectric point itself.

According to Harris [1923] the simplest mode of reaction of formaldehyde with an amino-group will lead to the formation of a methylene-imino-linkage, such reaction, however, not going to completion but attaining an equilibrium.

The results obtained, probably owing to the existence of the equilibrium

just referred to, were not as conclusive as had been hoped. They may be very briefly summarised as follows.

(a) In solutions of denatured oxyhaemoglobin containing formaldehyde (up to 0.5 %), some precipitation occurs on the acid side of the isoelectric point as far as p_H 3, and at the isoelectric point. There is no precipitation at p_H 7 and at higher p_H values.

(b) In solutions of denatured oxyhaemoglobin not containing formaldehyde there is complete precipitation at the isoelectric point and within a certain range on either side thereof, *i.e.* precipitation is still practically complete even at p_H 8 and decreases as the solution becomes more alkaline, whilst on the acid side the precipitate is still fairly complete even as far as p_H 3. The range over which precipitation occurs is greater in the absence of formaldehyde than in its presence.

As in the case of denatured oxyhaemoglobin the p_H range and degree of precipitation of denatured egg-albumin are both decreased by the addition of formaldehyde, and the greater the amount of formaldehyde added the greater the decrease.

The behaviour of both proteins towards formaldehyde may therefore be taken as evidence in support of the view that flocculation involves the amino- (and consequently the carboxyl) groups.

SUMMARY.

1. The unimolecular velocity constants of the denaturation, by means of acid, of the two proteins oxyhaemoglobin and egg-albumin, have been determined at various temperatures and over a considerable p_H range.

2. From the observed velocity constants the corresponding values of the critical increments of activation for different temperature ranges have been calculated. It is found that for the acid denaturation of oxyhaemoglobin the critical increment has an average value of 12,000 cal. For egg-albumin the corresponding quantity lies between 36,000 and 48,000 cal.

3. Attention is drawn to the marked difference in the magnitude of the critical increments recorded and those already obtained for so-called heat denaturation, namely, 77,000 cal. for oxyhaemoglobin and 130,000 cal. for egg-albumin. Whether the values for acid denaturation merge into those for heat denaturation as the acidity and temperature range are altered to those usually associated with heat denaturation is under investigation.

4. The influence of formaldehyde upon the flocculation of denatured protein has been examined. It is found that the p_H range over which precipitation occurs is greater in the absence of formaldehyde than in its presence. This serves to confirm the generally accepted view that flocculation involves the amino- (and consequently the carboxyl) groups.

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