XXI. THE DENATURATION OF PROTEINS.

VI. THE TITRATION OF THE BASIC AND ACIDIC GROUPS IN EGG-ALBUMIN.

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INTRODUCTION.

THE present paper contains an account of work carried out to determine, with a greater degree of precision than has been hitherto possible, whether or no there is any change in the number of free amino- and carboxyl groups in the protein on denaturation.

Let us first consider the work carried out by P. S. Lewis [1927] in this laboratory in which he determined the number of free carboxyl and amino-groups in haemoglobin before and after denaturation. His method was to add a known amount of standard acid or alkali to a definite volume of protein solution of known strength, and then to measure the p_H . The p_H was then plotted against the amount of acid or alkali added, for denatured and undenatured protein respectively. He found that the curves were identical, and so concluded that there was no change in the number of free amino- and carboxyl groups as a consequence of denaturation. However, in the light of later work by Cubin [1929], also in this laboratory, on denaturation at low temperatures and low p_H values, it appears that the concentration of acid in most of P. S. Lewis's experiments was sufficient to denature the haemoglobin during the time taken in carrying out the titration process itself. Thus from Cubin's results of the rate of denaturation at 25° , the temperature at which P. S. Lewis performed his titrations, it is found that denaturation takes place below p_H 4.0 almost instantaneously. The titration curve obtained by P. S. Lewis depends on eight points, three of which are above p_H 4 and five below. The titration is complete at a p_H of about 2.2. Hence we must conclude that in his measurements the amount of acid added for complete titration (obtained from the more acid end of the curve) was the same because he was in each case dealing with denatured protein. This fact has been confirmed by the writer in the following way. A haemoglobin solution of the strength used by P. S. Lewis was kept at 25° in the same quantity of 0.096 *N* acid and for the same length of time as employed by him. The solution was rapidly cooled

to 0°. By addition of ammonia the p_H was adjusted to the isoelectric point when the protein flocculated. Hence the titration curves obtained by P. S. Lewis below p_H 4 (the most important region) were identical because he was in each case inadvertently titrating denatured material.

In the case of his alkali titrations it is not possible to speak with such certainty. Assuming, however, that the action of alkali at a certain distance on one side of the neutrality point is just as great as that of acid at the same distance on the other side (certainly true for p_H values close to the neutrality point), we infer that over part of the titration range the material was denatured in both cases. It is only fair to this investigator to point out that this criticism of his titration conditions only becomes possible in the light of the later work by Cubin.

As regards the chemical nature of denaturation several possibilities, plausible on general grounds, have been criticised on the score that they would involve a change in the number of free carboxyl and amino-groups in the protein molecule on denaturation. Hence it is obviously of considerable importance to attempt to determine whether this change does or does not occur. The only means of doing this for haemoglobin would be by working at sufficiently low temperatures. The lowest temperature at which it would be possible to work is 0°, and the writer has calculated the values of the velocity constants of denaturation of haemoglobin at this temperature over the significant range, viz. $p_H < 4$, assuming the critical increment 11,000 calories obtained by Cubin for 18° to 25° to be valid for the range 18° to 0°. It is found that the amounts of acid used are sufficient rapidly to denature the protein at this temperature during the time of the experiment. Thus at p_H 3.5, 5% of the haemoglobin would be denatured in ten minutes, whilst at p_H 3.0 denaturation is practically instantaneous.

It appears possible that another protein, egg-albumin, might be a more suitable material to use in these experiments, in view of the fact that denaturation in the acid region is much less easily brought about than it is in the case of haemoglobin. Accordingly the writer has carried out these titrations using crystallised egg-albumin. The egg-albumin was prepared by the method of Hopkins and Pinkus as modified by Sørensen [1917]. The estimation of the albumin content was made by the method of Devoto as given by Hopkins [1900].

TITRATION OF EGG-ALBUMIN WITH ACID.

The method employed in these experiments was that used by Harris [1923, 2, 3; 1924] for the titration of various amino-acids and complex electrolytes, and adopted by P. S. Lewis [1927] for haemoglobin.

The method is to determine the p_H reached when a known volume of standard acid is added to a definite volume of protein solution of known strength. A series of readings is taken, corresponding to the addition of varying volumes of the acid. Thus a curve can be plotted of p_H attained against

volume of acid added. In a similar way a curve can be obtained of the p_H attained by the solvent against volume of acid added. This serves as a blank correction curve. Subtracting the volume of acid required to bring the solvent to a certain p_H from that required to bring both solvent and protein to this p_H , one obtains the volume of acid combined with the protein. The curve obtained by plotting p_H against acid combined with protein at a series of p_H values is asymptotic to the p_H axis when sufficient acid has been added to complete the titration. Experiments of this nature were performed using undenatured and denatured egg-albumin respectively. The titration in each case starts from the neutrality point of water.

All measurements were carried out in a thermostat regulated to 25°. In order to guard against denaturation as a result of prolonged exposure to acid, each point on the curve was obtained as the result of a separate experiment. Each result shown is the mean value of several (generally four) readings. The denaturation was performed by warming the solution (at p_H 6.99 in order to prevent change of p_H due to hydrolytic scissions [cf. P. S. Lewis, 1926]) in a corked flask in the steam of a briskly boiling water-bath. After denaturation was complete the cooled flask was tilted in order to collect the moisture which had condensed on the sides of the vessel.

The p_H was determined by the quinhydrone electrode, following the technique of Corran and Lewis [1924], the cell employed being of the type:

Gold foil	Solution under test saturated with quinhydrone	Saturated KCl	Normal KCl saturated with calomel	Mercury
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The solution used contained 1 g. of egg-albumin and 0.203 g. of ammonium sulphate per 100 cc. The blank solution contained 0.203 g. of ammonium sulphate per 100 cc. The acid used for titration was 0.0905 *N* sulphuric acid.

The p_H readings are accurate to p_H 0.01, and the titres to 0.005 cc.

The following are the results obtained (Tables I, II, III and Fig. 1).

Table I. *Titration of 10 cc. 1 % egg-albumin with 0.0905 N sulphuric acid at 25°.*

Acid added to 10 cc. solution cc.	Mean p_H of blank	Mean p_H undenatured	Mean p_H denatured
0.00	6.96	6.94	6.95
0.05	4.04	—	—
0.10	3.41	5.68	5.69
0.20	3.04	4.78	4.76
0.30	2.79	4.12	4.13
0.50	2.60	3.51	3.53
0.75	2.42	3.08	3.10
1.00	2.27	2.72	2.71
1.50	2.13	2.33	2.33

TITRATION OF EGG-ALBUMIN WITH ALKALI.

The method employed in this section was the formaldehyde method of Sørensen [1907] for the titration of amino-acids. Normally the titration with alkali has to be carried to a very alkaline end-point in order to obtain complete

titration. The addition of a certain amount of formaldehyde before titration enhances the acidic nature of the protein, and titration is complete at a much lower p_H . Harris [1924] attributes this to the combination of the amino-acids with the formaldehyde to give methylene-imino-derivatives. As an alternative to formaldehyde a fairly concentrated solution of alcohol can be used in order to enhance the acidic nature of the protein. Owing to the fact that alcohol acts as a powerful denaturing agent this method cannot be adopted here.

Table II. *Corrected titres for undenatured egg-albumin.*

p_H	Titre (cc.)	Blank (cc.)	Corrected titre
6.94	0.00	0.00	0.00
5.68	0.10	0.008	0.092
4.78	0.20	0.026	0.174
4.12	0.30	0.048	0.252
3.51	0.50	0.092	0.408
3.08	0.75	0.183	0.567
2.72	1.00	0.363	0.637
2.33	1.50	0.860	0.640

Table III. *Corrected titres for denatured egg-albumin.*

p_H	Titre (cc.)	Blank (cc.)	Corrected titre
6.95	0.00	0.00	0.00
5.69	0.10	0.008	0.092
4.76	0.20	0.026	0.174
4.13	0.30	0.047	0.253
3.53	0.50	0.090	0.410
3.10	0.75	0.177	0.573
2.71	1.00	0.365	0.635
2.33	1.50	0.860	0.640

Fig. 1. Titration of egg-albumin with acid.

Curve 1, titration of solvent alone.

Curve 2, titration of undenatured and denatured albumin.

Curve 3, corrected titre for undenatured and denatured albumin.

It is known that in the presence of ammonium salts formaldehyde forms a mixture of methylamines, producing at the same time acids [Werner, 1917]. Thus with ammonium sulphate we get:

Hence it is not possible accurately to perform formaldehyde titrations in the presence of ammonium salts. In the present case it is not feasible to dialyse sufficiently long to remove all the ammonium salts. Although realising that true titration curves cannot be obtained by this method, the writer has performed the titration experiments to see if any noticeable difference in results occurred when using denatured in place of undenatured albumin.

In order to perform the titrations it is necessary to determine the p_{H} of a solution containing protein, salt, and formaldehyde when varying amounts of alkali are added. In order to guard against denaturation by alkali a fresh solution was made up before each reading. The glass electrode [Kerridge, 1925] was employed for measuring the p_{H} . If e_1 and e_2 are the E.M.F. measured using standard solution and solution of unknown p_{H} respectively, then

$$p_{\text{H}} = 3.97 + \frac{e_2 - e_1}{0.58}.$$

In general the results are accurate to 0.02–0.03 p_{H} . The titres are accurate to 0.005 cc.

By titrating against the solvent alone (*i.e.* water, ammonium sulphate and formaldehyde) a blank correction curve was obtained. Hence, by subtraction, the volume of alkali required to bring the protein to a definite p_{H} can be obtained. The results are given in Tables IV, V and VI, and the accompanying curves (Fig. 2) drawn from them.

Table IV. *Titration by 0.1357 N sodium hydroxide of 15 cc. of solution prepared from 10 cc. of 1% egg-albumin (initial p_{H} 7.08), and 5 cc. of formaldehyde solution (initial p_{H} 7.07).*

Alkali added (cc.)	Mean p_{H} of blank	Mean p_{H} undenatured	Mean p_{H} denatured
0.00	4.87	4.87	4.89
0.20	5.39	—	—
0.30	5.54	4.98	4.97
0.50	5.68	5.07	5.06
0.75	5.91	—	—
0.80	—	5.43	5.41
1.00	6.33	5.66	5.70
1.50	7.49	6.14	6.13

It will be observed that the actual p_{H} range covered in these titrations with alkali is 4.87 to 6.14, in spite of the fact that the separate solutions of formaldehyde and protein are initially at p_{H} 7.08. The acidity produced is due to interaction of formaldehyde with ammonium salt as already explained.

Table V. *Corrected titres for undenatured egg-albumin.*

p_H	Titre (cc.)	Blank (cc.)	Corrected titre
4.87	0.00	0.00	0.00
4.98	0.30	0.045	0.255
5.07	0.50	0.080	0.420
5.43	0.80	0.230	0.570
5.66	1.00	0.450	0.550
6.14	1.50	0.890	0.610

Table VI. *Corrected titres for denatured egg-albumin.*

p_H	Titre (cc.)	Blank (cc.)	Corrected titre
4.89	0.00	0.00	0.00
4.97	0.30	0.045	0.255
5.06	0.50	0.075	0.425
5.41	0.80	0.220	0.580
5.70	1.00	0.510	0.490
6.13	1.50	0.885	0.615

Fig. 2. Titration of egg-albumin with alkali.

Curve 1, titration of solvent alone.

Curve 2, titration of undenatured and denatured albumin.

Curve 3, corrected titre for undenatured and denatured albumin.

DISCUSSION.

Reviewing the above results we see that both in the case of acid and alkali titrations there is no appreciable difference in behaviour between undenatured and denatured egg-albumin. Such small differences as are manifest are attributable to experimental error. The question naturally arises whether the amount of acid or alkali added for titration was sufficient to denature the egg-albumin. In the case of acid titrations it can be quite definitely stated that this was not the case. At a temperature of 25° denaturation only proceeds at an appreciable rate when the p_H of the solution is below 1.75. Thus at p_H 1.72, 1.6 % is denatured in 2 hours, and at p_H 1.02 less than 10 % in 100 minutes. In the above experiments the titration curve becomes approximately asymptotic to the p_H axis when a p_H of 2.75 is reached. Hence there is no chance of appreciable denaturation occurring during the few minutes required for taking the reading.

In the case of titrations with alkali it is unlikely that any denaturation occurred as the p_H only alters from 4.9 to 6.15, in which region the protein is stable until a temperature considerably higher than 25° is reached. However the system here is very complex and it is not definitely known to what extent formaldehyde, formic acid, and the other reaction products present may bring about denaturation, and consequently it is not possible to speak with the same certainty.

Harris [1923, 1] states that he finds no evidence of any increase in the titration figure on denaturation, but gives no indication of how he arrived at such a conclusion. The writer's results agree, however, with this statement. More recently Hendrix and Wilson [1928] performed titration experiments on uncoagulated and heat-coagulated egg-albumin, and found that the uncoagulated protein combined with more acid and alkali than did the coagulated protein. Although their results appear to have been accurately performed it seems probable that their coagulated protein had been subjected to rather more severe treatment than simple denaturation followed by flocculation. In the first place they heated the protein at the isoelectric point, at which p_H hydrolytic scissions are known to accompany the denaturation process; an alteration in p_H will be observed due to this. Secondly they heat it in steam for half an hour, which seems to be an unnecessarily prolonged treatment at a p_H as low as 4.7. And thirdly they allow it to stand overnight before performing the titration experiments, thus allowing possible further decomposition of the protein, with a corresponding alteration in titratability. The writer considers these remarks to be justified by the fact that when Hendrix and Wilson subjected the egg-albumin to still more severe treatment, by washing with alcohol, drying in an oven at 60° , and pulverising, a still smaller ability to combine with acid and alkali was found. It is concluded that Hendrix and Wilson's results do not really bear upon the problem of simple denaturation.

The number of "free" titratable basic and acidic groups.

By the term "free" is meant the free amino- and carboxyl groups only in the protein molecule. From the titration curves it is calculated, assuming the molecular weight of egg-albumin to be 43,000 [Marrack and Hewitt, 1929], that the egg-albumin molecule contains 25.3 groups titratable by acid, and 36.5 titratable by alkali. It follows from the results of Osborne, Jones and Leavenworth [1909] that the following titratable amino-groups are present in egg-albumin:

histidine	...	11.0×10^{-5} mols.	} per g. albumin.
arginine	...	28.2×10^{-5} mols.	
lysine ...	...	25.7×10^{-5} mols.	

These values are calculated from the number of total nitrogen atoms, on the basis that one half of the total lysine-nitrogen, one third of the histidine-nitrogen, and one quarter of the arginine-nitrogen will be titratable. Hence

1 g. of egg-albumin contains 64.9×10^{-5} titratable amino-groups, or 27.8 titratable groups per molecule. This agrees reasonably well with the writer's titration value of 25.3 for titration with acid. Incidentally the number obtained by titration is not necessarily a limiting value even for free amino-groups.

Further, from the results of P. S. Lewis [1927] it can be calculated, assuming a molecular weight of 68,000 for haemoglobin [Adair, 1925], that there are 97.6 groups titratable by acid. From results by Van Slyke [1911] on the amount of histidine, arginine, and lysine present it follows that we should expect 99.3 titratable groups to be present. Thus in the case of both proteins we have good agreement between the calculated and "found" number of free groups titratable by acid. Owing to the absence of data with regard to the amounts of the various dicarboxylic acids present in egg-albumin and haemoglobin it has not been possible to compare the number of carboxyl groups with the number of groups titratable by alkali or by acid, the latter being a possibility on the zwitterion basis.

Fig. 3.

Curve 1, titration of undenatured albumin.

Curve 2, titration of denatured albumin.

a, a, limits of error.

b, b, position of curve for one extra titratable group.

The question now arises as to whether an increase or diminution of a very small number, say one, amino- or carboxyl group would be detected in the titration experiments in the case of egg-albumin. Let us consider the more accurate series of results, namely those involving titration with acid. The latter portion of the titration curve is plotted on the accompanying graph (Fig. 3) on a large scale, together with the limits of experimental error, and the

position of the end-point if one group more were present. From this graph it will be clearly seen that a change of one group titratable by acid would be detected by a perceptible alteration in the titration curve. Hence it can be definitely stated that no change in acid titratability accompanies denaturation.

The possible interpretation of the titration values.

Denaturation may be accompanied by a transition of the protein in part at least from the "ordinary" form into the zwitterion form. Such a hypothesis would at least be in agreement with the ease with which denatured material is flocculated. It is realised of course that zwitterion formation if it occurs is inadequate in itself to explain denaturation. Thus it does not explain the irreversibility of the process, and the high critical increment.

On the basis of the zwitterion theory, titration with acid involves a formation of un-ionised COOH groups thus:

Thus titration with acid is titration of $-\text{COO}^-$ groups, and in a similar way titration with alkali is titration of charged amino-groups $-\text{NH}_3^+$. However, any transition into the zwitterion form on denaturation if such occurs will not be revealed in the titration experiments for the following reason.

Let us suppose for the sake of clarity that prior to denaturation all the amino- and carboxyl groups are present in the "ordinary" un-ionised form at the isoelectric point, and that we have 25 $-\text{NH}_2$ groups and 36 $-\text{COOH}$ groups. Suppose that on denaturation some number not greater than 25, let us say arbitrarily 5 amino-groups, change over into the $-\text{NH}_3^+$ form. In order that zwitterions may be formed this must be accompanied by a change of 5 carboxyl groups into the form $-\text{COO}^-$. Hence we now have 20 $-\text{NH}_2$ and 5 $-\text{COO}^-$ groups titratable by acid. This is a total of 25 as was the case before denaturation, and hence we should not expect to find any difference in the acid titration curves before and after denaturation. In a similar way we have 36 $-\text{COOH}$ groups titratable by alkali before denaturation, and 31 $-\text{COOH}$ plus 5 $-\text{NH}_3^+$ groups titratable afterwards, so that again no difference in titration curves would be expected. This conclusion would hold even if the whole 25 NH_2 groups changed into the zwitterion form together, necessarily, with 25 COOH groups. Obviously this represents the maximum number of zwitterions that could possibly be formed in the case considered. Actually the case of titration by alkali is somewhat complicated by the presence of formaldehyde. The function of the formaldehyde is to combine with the basic group of the amino-acid (see equation (2) p. 167).

Harris [1929] has pointed out that on the old (as opposed to the zwitterion) view the apparent basic dissociation constant remains unchanged on addition of formaldehyde, while the apparent acidic dissociation constant increases, and that this effect is anomalous, as combination with the basic group should affect the basic constant. According to the new (or zwitterion) view the

apparent basic constant relates in actuality to the true acidic constant, and *vice versa*. Hence addition of formaldehyde causes a diminution of the true basic constant and leaves the true acidic constant unchanged. Thus the effect of formaldehyde no longer appears anomalous, and Harris cites this as evidence in favour of the zwitterion theory for amino-acids.

In the case of every amino-acid which is present to any extent in the zwitterion form there is an equilibrium between the absolutely un-ionised form and the zwitterion form:

On addition of formaldehyde the —NH_2 group reacts with it, while the ${}^+\text{NH}_3$ does not.

Thus, provided the dissociation constant of the group $\text{CH}_2 : \text{N—}$ is lower than that of —NH_2 , the addition of formaldehyde will lower the true basic dissociation constant of the amino-acid. The presence of the equilibrium (2) will naturally decrease the amount of zwitterion present in the solution, by moving the equilibrium (1) from right to left. For a given amount of formaldehyde added there will be a certain proportion of zwitterions, un-ionised amino-acid, and imino-compound. Addition of NaOH to this system will cause two reactions to take place simultaneously:

The latter will of course be followed by

We see that by either of the reactions (3), or (4) and (4a) (taken together), we arrive at the same system on addition of a certain amount of alkali. Hence the p_{H} attained by addition of a given amount of alkali will not be altered by the initial presence of a larger (or smaller) proportion of the zwitterion form. Hence we should not expect any difference in the alkali titration curves even if the denatured protein does exist in the zwitterion form to a greater extent than does the undenatured protein. To demonstrate the actual formation of zwitterions if such occurs in the act of denaturation will require a different type of experimental test.

SUMMARY.

1. It is shown that the titration experiments of P. S. Lewis with haemoglobin do not actually demonstrate that the titratability of undenatured and denatured proteins is the same, owing to the fact that the haemoglobin undergoes denaturation during the titration for all p_{H} values less than 4 and greater than 10. The present investigation is concerned with crystallised egg-albumin which can be titrated in the undenatured state owing to the relative slowness of denaturation even at p_{H} 1, and 25° .

2. Titration experiments with acid using the quinhydrone electrode, and with alkali using the glass electrode, have been carried out. It is found that in neither case does heat denaturation affect the number of titratable groups. It is shown that a change of one titratable group (if such change occurred) would be well beyond the limits of experimental error.

3. Using the value 43,000 obtained by Marrack and Hewitt for the molecular weight of egg-albumin, it is calculated that there are at least 25 groups "easily" titratable with acid and 36 similar groups titratable with alkali. The number of free amino-groups found by analysis is 27, which agrees with the writer's acid titration values.

4. It is shown that, if zwitterion formation does accompany denaturation, such a change cannot be detected by any alteration in titratability of the protein.

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