CXLIX. ON THE DENATURATION OF EGG-ALBUMIN BY WEAK ACIDS IN THE PRESENCE OF SALTS.

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THIS work arose out of an attempt to prepare a salt-free solution of the proteins of egg-white by means of electrolysis. It was soon found that egg-albumin itself was altered under the conditions of the experiment, and a substance was obtained which did not correspond in properties with any of the known denaturation products of albumin. It was thought, originally, that the change was due to the electric current itself, but subsequent investigation showed that the change was due to the joint action of the salts present, and the acid formed as a result of the electrolysis. The material may therefore be regarded as a stage in the denaturation of albumin by acids, and is of great interest, in that it is possible to obtain evidence as to its structural relationships to fresh albumin on the one hand, and to more fully denatured albumin on the other hand.

In the earliest experiments the egg-white was diluted with two volumes of water, beaten up and centrifuged to remove particles of membrane, etc. It was then introduced into an ordinary lamp glass, the bottom end of which was closed by muslin coated with collodion to form a semi-permeable membrane. A short length of wide glass tube, closed at the lower end in a similar manner, dipped into the liquid from above. This tube and a beaker surrounding the lower end of the lamp glass formed the electrode compartments of a tripartite cell, the lamp glass containing the albumin being the centre section. Under normal conditions the electrode compartments contained distilled water which could readily be removed in order to examine the substances passing through the membranes. A platinum anode was used at the top and a platinum or mercury cathode in the beaker: the membranes were about 15 cm. apart and 50 to 200 volts were used according to the conductivities of the solutions. It was expected that as soon as the electrolytes were removed, the globulin would be precipitated, leaving the albumin, "conalbumin" and ovomucoid for further treatment, but it was soon found that the precipitate was not entirely composed of globulin. The next step was to remove the globulin by half saturating the egg-white with ammonium sulphate, dialyse out most of the electrolyte and then submit the residue to electro-dialysis. When this was

done it was found that a considerable amount of the nitrogen passed through the membranes. Tyrosine was found abundantly at the anode in all cases.

In the earlier experiments with the globulin-free solution of the proteins a precipitate was formed during the electrolysis which was soluble in dilute alkalis (including ammonia) but insoluble in dilute acids. After the current had continued for some time, precipitation ceased. The proteins from the supernatant fluid could be precipitated by ammonium sulphate, precipitation commencing at about 45 % saturation and being completed at about 55 %. If this precipitated protein were dissolved in water, and the solution dialysed and again submitted to electrolysis, a further precipitation of the acidic protein occurred. The proteins remaining in the supernatant fluid could be precipitated by ammonium sulphate, and, after being freed from the greater part of this salt by dialysis, again yielded a further amount of the acidic protein on electrolysis. By repeating these processes, practically the whole of the albumins could be converted into the acidic protein. The results described above were not, however, constant, and it was finally found that if the albumin solution had been efficiently dialysed so as to remove very nearly all the ammonium sulphate, no precipitation took place on dialysis, although the albumin remaining in solution had undergone a change which is described below.

The three major constituents of the globulin-free egg-white were then examined individually. Ovomucoid was prepared in the usual way from two dozen eggs and then electrolysed for 8 days. No trace of turbidity was observed in the solution, although a trace of brownish material separated on the cathode membrane. The anode liquid only showed a trace of chloride and sulphate ions on the second day. The cathode membrane appeared to be slightly permeable during the first few hours and the cathode liquid became opalescent with a few white flocks visible: it reduced Fehling's solution and contained 25 mg. of nitrogen. During the remainder of the time (7 days) only 2.5 mg. passed through the membrane. The Hausmann numbers indicated a possible small decrease in amide nitrogen but otherwise no change.

Crystallised albumin and conalbumin appeared to behave in an identical manner, with the single exception that the finely divided conalbumin carries down more pigment and impurities than the crystalline albumin. A very lengthy series of experiments in several types of apparatus was carried out to determine the exact conditions for the formation of a precipitate, but for reasons which were clear later, these could not be found. In nearly all cases the albumin solutions were efficiently stirred during the experiments in order to prevent the albumin being carried to a membrane by cataphoresis and then being denatured by secondary actions at the membrane. The general results may be summarised as follows.

A pure electrolyte-free albumin solution shows no visible action when a current of a few milliamps is passed through it for 1 or 2 days in the cold. If now the solution and the water in the outer compartments are made $M/10$

with respect to ammonium sulphate and the current raised to 50 or 100 milliamps., which can be done without raising the temperature of the solution above 15°, no visible action occurs for several hours and then a rapidly increasing turbidity appears throughout the bulk of the solution and even when there is no stirrer it is not confined to the neighbourhood of the membranes. The following morning one-sixth or even more of the albumin may be centrifuged off as an insoluble fine powder. If the salt concentration is maintained, the whole of the albumin can be obtained in the altered form, but the action slows down as the albumin is removed from solution.

During the precipitation, fractions of a mg. of nitrogen pass through the membranes, but this appears to be entirely due to the passage of traces of protein. If the membrane is heated to 30° or 40° in water, it rapidly becomes permeable to the albumin. The Millon, glyoxylic acid and biuret reactions give negative results with the liquor from the electrode compartments. In the electro-dialysis before the addition of ammonium sulphate, no ammonia is liberated after the residual traces from the salt used in the preparation (about 0.05 % of the total) have been removed. During the prolonged electro-dialysis in presence of ammonium sulphate, the centre compartment becomes acid and addition of acetic acid to the albumin solution appears to hasten precipitation. A considerable number of experiments were carried out with 10 cc. of solution containing varying amounts of ammonium sulphate with or without acetic acid, but the necessary conditions for the formation of the precipitate soon after the closing of the electrical circuit could not be found owing to the large number of variables. A precipitate can also be formed without the aid of an electric current, although apparently the change is not so extensive (see p. 1182).

5 cc. of albumin solution containing 3.89 mg. of nitrogen per cc. were made up to 10 cc. with varying strengths of sulphuric acid and ammonium sulphate and allowed to stand overnight at room temperature with the following results:

H ₂ SO ₄	N/5			N/10			N/20		
	M/25	M/5	M	M/25	M/5	M	M/25	M/5	M
(NH ₄) ₂ SO ₄									
After 2 hours	+	+++	++	—	+	—	—	—	—
„ 3 „	+	++++	++	—	++	+	—	—	—
„ 22 „	+++	++++	+++	++	+++	+++	+	+++	++

The solutions were all clear at the start of the experiment: + signifies slightly turbid, ++ turbid to very turbid, +++ precipitate and ++++ much precipitate. After standing overnight the precipitates settled to the bottom leaving a clear supernatant liquid. A sample of this precipitate was prepared for analysis by allowing 50 cc. of albumin solution (3.89 mg. of nitrogen per cc.) made up to 100 cc. and M/5 ammonium sulphate and N/5 sulphuric acid to stand for 20 hours. The precipitate was then centrifuged, washed and made up to 100 cc. of suspension in water (preparation III A in table on p. 1182).

All the insoluble products obtained up to this point appear to be identical.

They are insoluble in water, soluble in dilute alkalis, including ammonia, and are reprecipitated from alkaline solution by acids, but if the temperature rises at all during the neutralisation they become insoluble in alkalis. In some cases, particularly when electrolysis has been prolonged, there is a small part which is not soluble in ammonia. The freshly prepared material dissolves readily, giving a perfectly clear solution. It is not soluble in dilute acids in the cold, but is soluble with alteration in $N/25$ HCl at 37° . After solution at 37° the substance has the properties of an acid albumin, only being reprecipitated round the isoelectric point. All samples of the insoluble product showed an intense nitroprusside reaction. Within the limit of experimental error, the Hausmann numbers of the insoluble products were identical with those of crystalline egg-albumin and "conalbumin."

Action of ferments on acid protein.

The effect of trypsin and pepsin on the acidic precipitated protein was examined. Liquor pancreaticus (Benger) was used for the tryptic titration. The curves (see Fig. 1) for the rate of digestion were identical for the altered product and coagulated albumin, while unheated albumin showed a slower rate initially but the final result was the same. It was difficult to examine the effects of pepsin as the substance dissolved in the hydrochloric acid used for the digestion. Some comparison could be made by using extremely dilute acid and comparing the turbidities in the usual way.

Fig. 1.

The results are given in tabular form, the amount of pepsin being in arbitrary units.

		Coagulated				Altered albumin							
Time in hours	HCl Pepsin	1.9	2.5	3	21	0.2	0.4	0.7	1.0	1.9	2.5	3	21
N/100	10	+++	C	—	—	+++	+++	C	—	—	—	—	—
	5	++	C	—	—	+++	+++	+++	C	—	—	—	—
	2	+	++	—	C	+++	+++	+++	C	—	—	—	—
	0		No action			—	+	+	+	++	++	++	++
N/250	10	—	—		++	—	—	+	+	+	++	+++	C
	5	—	—	—	++	—	—	+	+	+	++	+++	C
	2	—	—	—	+	—	—	+	+	+	++	+++	C
	0		No action			—	—	—	—	+	+	+	+
N/500	10	—	—		+	—	—	—	+	+	+	++	C
	5	—	—	—	+	—	—	—	+	+	+	++	C
	2	—	—	—	+	—	—	—	—	+	+	++	+++
	0		No action			—	—	—	—	—	—	—	+

C=complete

These results indicate that the acidic protein is more readily digested than coagulated protein.

Analysis and preparation of products.

Crystalline egg-albumin was prepared in the usual manner from 820 cc. of egg-white. It was then recrystallised twice and its solution dialysed for 11 days in running distilled water. It then contained 0.05 % of its nitrogen in the form of ammonia. It filtered clear (preparation I (a)). The coagulated albumin was prepared by heating a dilute solution of albumin for 20 minutes (preparation I (b)). 200 cc. of a solution of crystallised egg-albumin containing 4.4 mg. of protein N and 3.6 mg. of ammonium sulphate N per cc. were electrolysed for 2 days using 100 milliamps. during the day and 50 at night. The insoluble precipitate which formed was centrifuged off, washed thoroughly on the centrifuge and suspended in 100 cc. of water (preparation III B (a)). One part of the suspension was directly analysed and another part was heated for half an hour on the water-bath and then analysed (preparation III A (b)).

After centrifuging off the precipitate, saturated ammonium sulphate solution was added to the slightly turbid liquid, when a bulky gelatinous precipitate was formed, which occupied half the volume even after centrifuging. It did not dissolve quite completely in water and its solution was dialysed till free from ammonium sulphate and then filtered. In the table below it is referred to as "first fraction of albumin not precipitated during electrolysis with ammonium sulphate" (preparation II (a)). The greater part of the protein could be precipitated from this solution by 45 % saturation with ammonium sulphate. From the filtrate of this precipitate a second fraction could be obtained on addition of more ammonium sulphate. It did not form a coherent mass on centrifuging and was readily soluble in water. The aqueous solution was dialysed (preparation II (b)).

Experiments were also carried out to determine the effect of salts other than ammonium sulphate on the production of the acidic protein. For this purpose two exactly similar solutions of crystallised egg-albumin were electro-

lysed in series overnight to remove last traces of ammonium sulphate, and then one was made $M/10$ with potassium chloride and the other $M/10$ with ammonium sulphate. The electrolysis was continued with a current of 50–100 milliamps. The two liquids behaved differently when submitted to electrolysis. After 20 hours the solution containing ammonium sulphate gave a precipitate, which was centrifuged off. 5 cc. of the clear liquid required 3 cc. sodium hydroxide to change methyl red. The solution containing the potassium chloride, however, showed only a faint turbidity. 5 cc. required 2.5 cc. $N/10$ alkali for neutralisation to methyl red, and at the neutral point a heavy precipitate came down, which dissolved in excess of alkali. On continuing the electrolysis, a second precipitate was formed in the ammonium sulphate solution. No precipitate was formed in the experiment with potassium chloride, but on addition of half the volume of $4N$ potassium chloride, practically the whole of the protein was precipitated. The precipitate behaved exactly like the insoluble precipitate previously obtained, being soluble in dilute alkalis, insoluble in dilute acids and showing a strong nitroprusside reaction (preparation III A (c)). Another sample of salt-free albumin was electrolysed in the presence of $M/10$ sodium chloride. A flocculent precipitate formed overnight. The analyses of this sample are given with reserve as free chloride was found in the solution (preparation III B (b)).

Another sample of albumin solution free from electrolytes was electrolysed for 20 hours. No precipitate had formed and the clear solution was submitted immediately to analysis (preparation II (c)).

Preparations as numbered in text				cc. $N/10$ KOH	COOH groups	% amino-N	NH_2 groups	Nitro- prusside reaction
I. (a)	Crystalline albumin	...	...	2.39	25	4.14 4.44	16 17	—
(b)	Coagulated albumin	...	...	2.25	24	5.39	20	—
(c)	Conalbumin	...	...	2.57	27	5.52	21	—
II. (a)	First fraction of albumin not pptd. during electrolysis with $(NH_4)_2SO_4$			3.55	38	4.84 5.57	18 21	—
(b)	Second fraction ditto	...	...	3.51	37	—	—	—
(c)	Albumin electrolysed without electro- lytes	...	...	3.55	38	—	—	+
III. A (a)	Pptd. with $M/5 (NH_4)_2SO_4$ $N/5 H_2SO_4$ in the cold			4.16	44	3.42	13	+
(b)	Electrolysed with $(NH_4)_2SO_4$ and heated $\frac{1}{2}$ hour			4.13	44	—	—	+
(c)	Electrolysed with KCl			4.48	48	3.61	14	+
B (a)	Electrolysed with $(NH_4)_2SO_4$			5.42	58	4.20* 4.66	16 18	+
(b)	Electrolysed with NaCl			5.52	59	—	—	+
(c)	Conalbumin electrolysed with $(NH_4)_2SO_4$			5.81	62	—	—	+

* Is a different sample prepared at a different time.

Analysis. The solutions or suspensions were examined for free amino-groups by the ordinary Van Slyke method. Two portions of 10 cc. were each shaken for $\frac{3}{4}$ hour before the total nitrogen evolved was measured. The free amino-nitrogen is expressed as a percentage of the total and also as free

amino-groups per molecular aggregate assuming Sørensen's value of 34,000 and 380 nitrogen atoms. The free carboxyl groups (or other acidic groups) were estimated directly by the formaldehyde titration method or by direct titration in alcoholic suspension. The titration in alcoholic suspension using *N*/10 alcoholic potash gave slightly lower readings, but the figures were relatively the same. In the formaldehyde titration 45 cc. of solution or suspension containing 50 mg. of protein nitrogen were mixed with 40 cc. of formalin diluted with an equal volume of water. The results are given as cc. of *N*/10 alkali for 50 mg. of nitrogen and also calculated as free carboxyl groups per molecular aggregate. The nitroprusside reaction was carried out as described by Harris [1923]. It was not found possible to determine the free —SH groups quantitatively as the ordinary oxidation methods are not applicable owing to the insoluble character of the substances and to the fact that the large molecular aggregates prevent quantitative conversion to the disulphide owing to steric factors.

DISCUSSION.

The number of free carboxyl groups divides the substances examined into three groups:

I. 24–27 free carboxyl groups and negative nitroprusside reaction; includes crystalline albumin, coagulated albumin and conalbumin.

II. 37 or 38 free carboxyl groups and negative nitroprusside reaction; includes the three materials which have been electrolysed but not converted into an insoluble form: this group is not due to admixture with members of group III, but must be regarded as a definite intermediate stage as there is no nitroprusside reaction.

III. Characterised by the nitroprusside reaction and by the fact that all members are insoluble in water and dilute acids. It can probably be subdivided into two sub-groups, the one containing 44 to 48 carboxyl groups and the other 58 to 62. The latter is probably only a later stage of the former. The three fully electrolysed products form the second class (sub-group III (*a*)) and the first is composed of the material obtained by acid and salt without electrolysis on long standing, a fully electrolysed material heated on the water-bath and also the material electrolysed with potassium chloride which did not precipitate or become insoluble until after salting out with more potassium chloride. There are thus nearly two and a half times as many free carboxyl or other acid groups in the denatured albumin as in the original crystalline albumin.

The values for the free amino-groups exhibit no regularities and with two exceptions lie between 16 and 21. The experimental error in these experiments is great owing to the difficulty of transferring the suspensions completely to the apparatus, and the error is greatest with the denser precipitates in group III, since a relatively larger proportion cannot be washed into the apparatus. Both the low results, 13 and 14, were obtained with dilute suspensions, when

the error was even more serious. Whether there is any closing up of NH_2 groups or not, it is quite clear that the increase in free carboxyl or other acidic groups is not due to the breakdown of peptide linkings which would be accompanied by an *increase* in amino-groups. The latter stage of the change is certainly accompanied by the unmasking of free thiol groups and the process may be formulated

that is, as the breaking down of a thiol-depside ring. It is not possible to say whether the free thiol group would behave as an acidic group and be titrated in the formaldehyde titration as it would depend on the other parts of the molecule, but it is probable that it would act in this way. Thioglycollic acid is a normal dibasic acid and forms normal salts $\text{NaS} \cdot \text{CH}_2 \cdot \text{COONa}$. The members of group III show as many as 22 more free acid groups than group II and this would require 11 thiol groups to be liberated, *i.e.* equivalent to 1 % of the total albumin as thiol sulphur. The cystine in albumin is insufficient to account for this quantity and it would have to be derived from the unidentified sulphur in the albumin. Harris considered this explanation of the production of the free thiol groups in denatured protein but inclined to another formulation; in the present case only the one explanation is valid. It is not a complete explanation as it does not take into account the formation of group II from group I and the opening up of ten acid groups. It is an interesting speculation to imagine that this is due to the opening of similar depside rings and liberation of hydroxyl groups instead of thiol groups. The rapid increase in the number of hydroxyamino-acids which have been isolated is of interest in this connection and further it is known that tyrosine is one of the first, if not the first, amino-acid to be liberated from albumin under the influence of enzymes. The natural extension of this work to acid denatured proteins is being carried out in these laboratories.

It has been found that similar acidic substances, yielding the nitroprusside reaction, are formed when egg-albumin is denatured at the interfaces of aqueous egg-albumin solutions and fluids such as chloroform which are immiscible with water, the action of which is very specific. The work on these phenomena will form the subject of a later communication.

SUMMARY.

- (1) A well-defined acidic denatured product of albumin has been obtained in various ways. It is soluble in dilute alkalis and insoluble in dilute acids.
- (2) The formation of this substance involves an increase in the number of free acid groups without corresponding increase in the amino-groups. At the same time free $-\text{SH}$ groups appear.

(3) Evidence is produced to show the presence of a new type of linkage in proteins. A *thiopeptide* or C—CO.S—C— linkage breaks down to give the corresponding carboxyl and thiol groups.

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REFERENCE.

Harris (1923). *Proc. Roy. Soc. Lond. B*, **94**, 426.