author early in 1948, at Harvard University. The author wishes to express his appreciation to Prof. J. J. Lingane, both for his generous provision of materials and facilities at that time, and for a number of very helpful discussions.

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[CONTRIBUTION FROM THE DEPARTMENTS OF BIOCHEMISTRY AND MEDICINE, COLLEGE OF PHYSICIANS AND SURGEONS, COLUMBIA UNIVERSITY]

The Deamination of Crystalline Egg Albumin.[1,2] I. Preparation and Properties of Various Soluble and Denatured Derivatives

BY PAUL H. MAURER AND MICHAEL HEIDELBERGER

The removal of 27 to 36% of amino groups from crystalline egg albumin (Ea) under mild conditions leads to a soluble deaminated Ea. At the same time a denatured derivative is formed and in this the extent of deamination is greater than in the soluble fraction. Upon further deamination the soluble deaminated Ea becomes insoluble at its isoelectric point, indicating that removal of this portion of the free $-NH_2$ groups which may participate in holding the native molecule in corpuscular form leads to partial unfolding of the molecule. Complete deamination was not achieved.

The immediate purpose of the present investigation was to determine whether or not crystalline egg albumin (Ea) could be deaminated without being denatured. Since the very meaning of the term "denatured" is under dispute, it was felt that, in spite of the large number of investigations on denatured proteins,[3] a careful study of the deamination of this easily irreversibly denatured protein might lead to additional knowledge of protein structure and the changes involved in denaturation. Because of the drastic conditions employed in previous deamination studies[4,5] it appeared that a deaminated undenatured Ea had never been prepared. For the purposes of the present papers, denaturation is defined as the conversion of the protein used, soluble in the native state at its isoelectric point, into a form or forms insoluble at their isoelectric points, a definition which has been shown to be useful in previous studies from this laboratory.[6–8]

Experimental

1. **Preparation of Ea and Its Derivatives.**—The Ea, prepared and five times recrystallized by the method of Kekwick and Cannan,[9] was dissolved in H_2O and dialyzed against water in the presence of toluene until sulfate-free, and allowed to stand at room temperature until all insoluble material settled, leaving a water-clear supernatant.[8]

The following conditions for deamination were found advantageous: Ea was treated in 0.5 M acetate buffer at pH 4.0,[10] with M $NaNO_2$ for varying periods at 0–3° (Table I). The reaction was stopped by careful neutralization with 2 N NaOH to pH 7.5. After dialysis until free from nitrite the solution was treated with neutralized thioglycolic acid at a final concentration of 1 M at 0–2° for 18 hours[11] to reduce any –S–S– linkages formed by the oxidizing action of HNO_2.[12,13] The solution was dialyzed free from thioglycolate as tested for by $CoSO_4$,[14] and 1-ml. aliquot portions were

TABLE I

PREPARATION AND PROPERTIES OF Ea, DEAMINATED Ea, AND DENATURED Ea

Preparation	Conditions for preparation: Reaction mixture	Temp., °C.	Time, hr.	Rela. proportions of A(1.0) and B	Deamination, %, ninhydrin method
Ea	Ref. (9)				0[a]
3A	pH 4.0 OAc^-, NO_2^-	0–3	6	1.0	33
3B	pH 4.0 OAc^-, NO_2^-	0–3	6	1.4	27
3B (A)	pH 4.0 OAc^-, NO_2^-	0–3	8 more		49
3B (B)	pH 4.0 OAc^-, NO_2^-	0–3	8 more	[b]	27
4A	pH 4.0 OAc^-, NO_2^-	0–3	8	1.0	42
4B	pH 4.0 OAc^-, NO_2^-	0–3	8	1.3	31
5A	pH 4.0 OAc^-, NO_2^-	0–3	17.5	1.0	56
5B	pH 4.0 OAc^-, NO_2^-	0–3	17.5	0.4	36
6A	pH 4.0 OAc^-, NO_2^-	0–3	7.5	1.0	42
6B	pH 4.0 OAc^-, NO_2^-	0–3	7.5	0.9	29
8A	pH 4.0 OAc^-, NO_2^-	0–3	8		44
8B	pH 4.0 OAc^-, NO_2^-	0–3	8		36
10A	pH 4.0 OAc^-, NO_2^-	R.T.[c]	18.5		80
5A Dn	pH 1.5 HCl	R.T.	72		
6A Dn	pH 1.5 HCl	R.T.	72		
8A Dn	pH 1.5 HCl	R.T.	144		
6B Dn	pH 1.5 HCl	R-T.	96		
8B Dn	pH 1.6 HCl	R.T.	144		
DnEa 105	pH 1.5 HCl	R.T.	72		
DnEa 105 Deam (SH)	pH 3.0 HNO_2	0–2	18		
DnEa 105 Deam (SS)	pH 3.0 HNO_2	0–2	18		38
DnEa 106	pH 1.7 HCl	R.T.	72		
DnEa 106 Deam (–SH)	pH 3.5 HNO_2	0–2	17		
DnEa 106 Deam (SS)	pH 3.5 HNO_2	0–2	17		58
8A FNA	pH 3.5 HNO_2	0–2	17		67
Ea FNA	pH 3.5 HNO_2	0–2	16		56

[a] Ratio of amino N/total in Ea found, 0.045; as also in Ref. (15). [b] Very little isolated. [c] R.T. = room temperature.

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(2) Presented before the 40th Annual Meeting of the American Society of Biological Chemists, Detroit, Michigan, April 18–22, 1949, and before the 41st Annual Meeting of the American Society of Biological Chemists, Atlantic City, New Jersey, April 17–21, 1950.

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added to 3 ml. of 0.1 *M* citrate HCl buffer at 0°.[7] After centrifugation the N in the precipitates formed was estimated by the micro-Kjeldahl method,[15a] with results as shown in Table II. The entire mixture was then fractionated at the *p*H of maximum precipitation, 3.9 to 4.1. The ratios of the yields of A and B after varying periods of time are shown in Table I, column 5, the quantity of A formed being taken as 1.0 in each case.

TABLE II

FRACTIONATION OF A AND B MIXTURE

*p*H	% total N precipitated from 4A and 4B mixture
3.2	38.4
3.7	43.5
4.1	44.3
4.2	42.5
5.0	35.0

The insoluble fraction, A, was redissolved and reprecipitated several times at its isoelectric point until no more soluble protein, B, could be detected in the supernatant by heat coagulation or reaction with antiserum to Ea (see Paper III). The *p*H of both fractions was adjusted to 7.5 and the solutions were stored in the ice-box with toluene. Treatment with thioglycolate was sometimes omitted in order to obtain the -S-S- forms.

Acid denatured egg albumin (DnEa) prepared according to Ref. (8) was chosen as a basis for comparison with fraction A. Since differences quickly came to light, attempts were made to denature A and B by means of acid at *p*H 1.5. Fraction A underwent little change while B was more nearly quantitatively denatured than is Ea itself.[8] These ADn and BDn preparations were worked up as described for acid DnEa (8).

To obtain a possible "isomer" of BDn, acid DnEa was deaminated. The acetate buffer could not be used because of the insolubility of acid DnEa at *p*H 4.0 and the aggregating effect of the salt. Free HNO_2, prepared by mixing equal quantities of *M* H_2SO_4 and *M* $Ba(NO_2)_2$ at 0° and centrifuging in the cold, was poured into a DnEa solution (5 mg. N/ml.) at 0°. A drop of caprylic alcohol was added to reduce the initial foaming. The *p*H was adjusted to 3-3.5. At the end of the reaction period (Table I) the mixture was diluted with cold water before neutralization with 0.1 *M* NaOH so that the salt concentration would not exceed 0.02 *M* at the *p*H of maximum precipitation, 4-4.2.[8] The protein was redissolved at *p*H 7.5 and reprecipitated 5 to 6 times at the isoelectric point to remove the nitrite. The deaminated oxidized acid DnEa, -S-S- form, was treated at *p*H 7.5 with neutralized thioglycolate as previously described. Both the deaminated DnEa -S-S- and -SH forms were redissolved at *p*H 7.5 and stored in the ice-box with toluene.

This deaminating procedure was also applied to native Ea (Ea FNA) and Fraction 8A (8A FNA) (Table I).

2. **Amino N.**—In the initial products amino N was determined both by the Van Slyke manometric procedure[16] with 30 minutes of shaking, and by the ninhydrin colorimetric method,[17] but after they were found to yield similar results later preparations were analyzed only by the ninhydrin method. Readings were made in a Coleman spectrophotometer at 570 mμ.[18]

Results and Discussion

From Table I it is seen that with increased time of exposure to deamination the relative amount of insoluble fraction A increased. However, the extent of deamination increased more in fraction A than in fraction B, remaining relatively constant in the latter at 27-36 per cent. Fraenkel-Conrat[12] briefly mentioned a similar result, stating[13] that deamination at 0° for one hour gave 23% of insoluble product which was deaminated 45%, whereas the soluble fraction had lost only 17% of its $-NH_2$ groups.

To determine whether the insoluble protein was formed through an intermediate soluble deaminated Ea, fraction 3B was further exposed to the deaminating mixture. An insoluble A fraction 3B(A), was obtained at the expense of B 3B(B), but increased deamination was noted only in A. Apparently deamination beyond 27-36 per cent. leads to a denatured protein.

In order to determine whether the salt concentration and *p*H used were leading to denaturation, Ea was exposed for 18 hr. at 0-2° to the same acetate buffer with *M* $NaNO_3$ instead of $NaNO_2$. No denatured Ea was obtained at the isoelectric point of Ea. Ea is ordinarily stable at *p*H 4.0[19] and even at *p*H 2.6 at 0°.[20] However, deaminated fraction B is less stable, since it deposited about 10% of protein when treated with $NaNO_3$ as above.

The findings are in accord with the theories of Mirsky and Pauling[21] and Eyring and Stearn[22] concerning some of the forces holding the protein in the corpuscular form. Removal of the easily available $-NH_2$ groups on the surface of the molecule leads to a protein differing little from native Ea except in a greater susceptibility to influences leading to denaturation. However, removal of further $-NH_2$ groups which may have the function of participating with free -COOH groups in holding the protein in the native globular form by hydrogen bond formation or salt linkage leads to an unfolding of the molecule, as will be shown in the following papers. The resulting more asymmetric protein possesses chemical, physical and immunological properties resembling those of proteins in the denatured state.

Jirgensons[5] ascribed a similar role to the $-NH_2$ groups as a whole, not recognizing that some of them could be removed without changes in molecular shape. His treatment of the preparations would have resulted only in denatured proteins, as shown by the following: Deamination at *p*H 4 and room temperature (Preparation 10A) led to a deaminated Ea which was insoluble at its isoelectric point, and only 35% of which was soluble at *p*H 7.5. In a control experiment with $NaNO_3$ instead of $NaNO_2$ 30% of the Ea used was rendered insoluble.

The incomplete removal of the $-NH_2$ groups may be due to one or more of three distinct effects. The first is that the ε-amino groups of lysine, which account for practically all of the free amino groups in the Ea molecule[9,23,24] react very slowly with HNO_2 at 0-5°.[25] The second may be the unreactivity and unavailability of some of the $-NH_2$ groups due to the structure of the protein,[26] an

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aspect of the problem which has been studied in proteins such as Ea, β-lactoglobulin and horse serum pseudoglobulin.[27] Only 70% of the amino groups of Ea are affected by PhNCO[28] and HCHO, whereas acetic anhydride blocks 85% of the amino groups.[13] Brand, *et al.*, noted a difference in the rates of deamination of proteins by HNO_2.[29] Differences in reactivity of the $-NH_2$ groups toward formalin have been observed with diphtheria toxin[30] and tobacco mosaic virus.[31]

The third factor influencing the removal of amino groups may be the *p*H. Kornblum and Iffland have shown that aliphatic primary $-NH_2$ groups[32] do not react below *p*H 3.0, indicating a narrow *p*H zone in which deamination is achieved. This probably explains the smaller extent of deamination of acid DnEa at *p*H 3.0 than at *p*H 3.5 (Table I).

Since more groupings are detectable in the denatured than in the native state,[33] it was hoped to obtain a highly deaminated protein by reacting free HNO_2 with acid DnEa at *p*H 3.0–3.5. However, deamination merely equalled that of Ea under the same conditions, but was greater than the deamination of Ea in the milder buffer mixture. Even 8A which had been deaminated 44% by the buffer mixture lost only an additional 23% of its $-NH_2$ groups after exposure to free HNO_2.

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The Deamination of Crystalline Egg Albumin.[1,2] II. Physical and Chemical Properties of the Soluble and Denatured Derivatives

BY PAUL H. MAURER, MICHAEL HEIDELBERGER AND DAN H. MOORE

The partially deaminated (27 to 36%) and soluble derivative of egg albumin, fraction B, has physical and chemical properties like those of native Ea and may therefore be termed an undenatured deaminated Ea. The insoluble derivative, fraction A, formed during deamination (33–56%) of Ea has many properties characteristic of the denatured state, but its configuration appears less extended than that of acid denatured Ea (DnEa) as indicated by nitroprusside tests, sedimentation and diffusion constants, and the reduced tendency to aggregate in salt solutions. This less completely unfolded protein is relatively stable to further treatment with acid at *p*H 1.5 whereas the B fraction is readily transformed into an acid DnEa derivative different from the one obtained by deaminating acid DnEa.

In the preceding paper[3] the preparation of various deaminated and denatured derivaties of crystalline egg albumin (Ea) was described. Since the solubility,[3] optical activity, viscosity, diffusion constant, sedimentation constant, electrophoretic mobility, ion binding and sensitivity to salt are all useful for the characterization of the protein molecule and detection of changes, these properties were studied.

1. **Optical Activity.**—The specific rotation of the protein solution was determined at room temperature, 22–25°, at about 1% concentration, after dialysis against phosphate buffer at *p*H 7.5, 0.05 ionic strength. The factor 6.45[4] was used to convert mg. N to mg. protein. An increase in negative rotation of Ea upon denaturation (Table I) is in accord with previous reports on this[5,6,6a] and other proteins[7], and has been ascribed to changes in the asymmetry of the "backbone" of the peptide chain[6,8] but may also be due to an unfolding of the molecule, as appears to be the cause of the parallel increase in ultraviolet absorption[9] and in the number of detectable -SH groups.[10]

The $[\alpha]_D$ of the soluble, or B, fractions resembled that of native Ea. After acid treatment of B at *p*H 1.5 $[\alpha]_D$ was like that of acid denatured Ea (DnEa) and of the A, or insoluble, deaminated fractions. No detectable change was observed after further acid treatment of A (Table I) nor was there significant change in the $[\alpha]_D$ of DnEa upon deamination (DnEa Deam -S-S-, for the oxidized form; -SH, for the reduced form (Table I)).

2. **Viscosity.**—Eight to ten ml. of a 1% protein solution were dialyzed at 0 to 5° for 4 to 5 days in the presence of toluene against two changes daily of 0.02 *M* phosphate buffer at *p*H 7.5, ionic strength 0.05, a concentration sufficient to suppress the electroviscous effect.[11] Viscosity determinations were made with 5 ml. of dialyzed solution in an Ostwald–Fenske type viscometer at 25 ± 0.5°.

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(2) Presented before the 40th Annual Meeting of the American Society of Biological Chemists, Detroit, Michigan, April 18–22, 1949, and before the 41st Annual Meeting of the American Society of Biological Chemists, Atlantic City, N. J., April 17–21, 1950.
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