tion through an intramolecular reaction analogous to the well-known intermolecular Michael reaction. Unlike the latter, however, the intramolecular reaction is not inhibited by alkyl substituents on the α- and β-carbon atoms of the acceptor group, and it appears to go substantially to completion. The products are derivatives of 2-carboxycoumaran-3-acetic acid.

The reaction was devised and is being further studied as a possible route to synthetic compounds related to morphine.

MINNEAPOLIS, MINNESOTA RECEIVED DECEMBER 29, 1944

[CONTRIBUTION FROM THE DEPARTMENTS OF BIOCHEMISTRY AND MEDICINE, COLLEGE OF PHYSICIANS AND SURGEONS, COLUMBIA UNIVERSITY, AND THE PRESBYTERIAN HOSPITAL, NEW YORK CITY]

Denatured Egg Albumin.[1] I. The Preparation and Purification of Crystalline Egg Albumin Denatured in Various Ways[2]

BY CATHERINE F. C. MACPHERSON AND MICHAEL HEIDELBERGER

Nowhere is the need for more precise information on denatured proteins more urgently implied than in the all-inclusive definition of denaturation given in the most recent review of the subject.[3] The prevailing utter lack of agreement as to the criteria of denaturation would seem to render a descriptive approach to the problem permissible, the more so as the original object of the present studies, the use of acid-denatured egg albumin (DnEa) as an intermediate, was thwarted by the extreme difficulty of obtaining successive preparations with reproducible properties. These difficulties, in part induced by the desire to avoid, as far as possible, degradation[4] of the egg albumin (Ea) used, necessitated a critical study of the preparation of acid-DnEa, and to this was added a similar investigation of other methods for the denaturation of Ea and the comparison and attempted correlation of the chemical, physical, and immunological properties of the products.

The criterion of denaturation employed was the insolubility, at the isoelectric point, of the product, DnEa, derived from the isoelectrically soluble native protein, hen egg albumin.

Qualitative immunological studies by Wu, Ten Broeck and Li[5] on egg albumin (Ea) denatured by acid, alkali, heat, and alcohol showed the denaturation products to be closely related to each other except that only alkali-DnEa did not react with antiserum to Ea and Ea did not react with antiserum to alkali-DnEa. Flosdorf and Chambers[6] found that denaturation by intense sound vibrations produced the same qualitative immunological changes as heating with acid or alkali. Mirsky[7] demonstrated that the same number of sulfhydryl groups was liberated in Ea denatured by urea, guanidine, Duponol P. C., heat, or shaking.

Each of these comparative studies dealt with only one property of DnEa and little attention was paid to the state of aggregation[8] or degradation[4] of the products, factors which not only would be expected to influence the chemical, physical, and immunological properties of DnEa but which also, if neglected, would cause confusion as to the changes attributable to denaturation alone. While it might be inferred from the few ultracentrifugal studies[9] on the particle weights of various kinds of DnEa that the molecules of these substances are associated or aggregated in water containing only just enough alkali to dissolve them, little, if anything, is known of the degree of association brought about by a particular denaturant or how this might be affected by variations in either the concentration of denaturant or the reaction time or temperature.

Experimental

Ea was prepared by the method of Kekwick and Cannan.[10] Four-times recrystallized Ea was air-dried and dialyzed against distilled water in the presence of toluene at 0–5° until no more sulfate was removed. Toward the end of these studies it was found that by allowing the salt-free isoelectric Ea solutions of varying opalescence to stand at 37° until all precipitate settled, any DnEa contained in them was removed, leaving a water-clear supernatant.

1. **Acid Denaturation of Ea.**—The denaturation of Ea by acid at a concentration of about 2.4 mg. Ea N per ml. was carried out by addition of 0.1 M HCl to bring the pH to 1.5 or 2.0. The final normality of the acid was usually about 0.05 or 0.025, respectively. The pH, determined with the glass electrode, remained constant during the reaction. In most instances the course of the de-

(1) A summary of this series of papers was read before a joint meeting of the New York and New Jersey Sections of the Society of American Bacteriologists on May 16, 1942, at Princeton, New Jersey.

(2) The work reported in this communication was carried out in part under the Harkness Research Fund of the Presbyterian Hospital and has been submitted by Catherine F. C. MacPherson in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Faculty of Pure Science, Columbia University. Original manuscript received July 6, 1944.

(3) H. Neurath, J. P. Greenstein, F. W. Putnam and J. O. Erickson, *Chem. Rev.*, **34**, 157 (1944).

(4) Degradation is here defined as the splitting off of nitrogenous or sulfur-containing portions of the labile protein molecule.

(5) H. Wu, C. Ten Broeck, and E. P. Li, *Chinese J. Physiol.*, 1, 277 (1927).

(6) E. W. Flosdorf and L. A. Chambers, *J. Immunol.*, **28**, 297 (1935).

(7) A. E. Mirsky, *J. Gen. Physiol.*, **24**, 725 (1941).

(8) Aggregation is defined as the linking of DnEa molecules, assuming M. W. DnEa = M. W. Ea = ca. 40,000, into aggregates or polymers. Opalescence and gel-formation were considered visual evidences of the process, useful for guidance during the preparative work. More substantial evidence is given in the text.

(9) (a) A. Rothen, *Annals N. Y. Acad. Sci.*, **43**, 229 (1942); (b) J. B. Nichols, THIS JOURNAL, **52**, 5176 (1930).

(10) R. A. Kekwick and R. K. Cannan, *Biochem. J.*, **30**, 232 (1936).

naturation was studied quantitatively at constant temperature.

Varying volumes of reaction mixture were pipetted out and added to 3 ml. of 0.1 *M* acetate buffer at *p*H 5 at 0°. Because 2 to 3 ml. of reaction mixture were needed for estimation of DnEa during the early stages, it was necessary to add a few drops of 0.1 *N* alkali to the buffer-protein solution in order to ensure isoelectric precipitation. All precipitates were washed twice in the cold with 3-ml. portions of buffer as already described.[11] In the process of washing precipitates of DnEa form films on the surface. These break into small fragments on mixing, and are partly lost when the supernatants are decanted, interfering with the accuracy of the analyses. The difficulty may be largely avoided by addition of saponin, up to 0.05%, to the buffer used for washing.[12] The validity of the method was established by preliminary tests which showed quantitative recovery of acid-DnEa from mixtures of solutions of Ea and DnEa in different proportions.

The rate studies permitted no clear-cut conclusions as to the order of the reaction. Approximately straight lines were obtained up to at least 50% conversion when either log [Ea] or 1/[Ea] was plotted against time. There was also considerable variation in the rates at which various preparations of Ea were denatured, as noted in Table I. Similar variation was observed recently for the action of urea on tobacco mosaic virus.[13]

TABLE I

COMPARISON OF RATES OF DENATURATION OF VARIOUS PREPARATIONS OF Ea BY ACID

DnEa prepn.	Ea prepn. used	*p*H at which denatured	Concn. NaCl, *M*	Temp., °C.	Reaction time, hr.	Extent of denaturation, %
17	7	1.96	0	25	46	50
22	8	2.01	0	25	21	50
16	7	1.51	0	26	6.8	50
21	8	1.53	0	25	5.5	50
	9	1.53	0	25	11.5	50
	1[a]	1.50	0.1	R. T.[c]	22[d]	60
	3[a]	1.50	0.1	R. T.	22[d]	94
	1[b]	1.96	0.5	R. T.	24.5[d]	26
	3[b]	1.96	0.5	R. T.	24.5[d]	44

[a] Simultaneous runs. [b] Simultaneous runs. [c] R. T. = room temperature. [d] Approximate.

Reaction mixtures of acid-DnEa were always opalescent, but the opalescence disappeared during aging after the products were isolated under the conditions recommended below. Such preparations were considered "normal." Lots of acid-DnEa showing unusual opalescence, or increasing opalescence during less cautious manipulation were listed as "aggregated."

2. **Alkali Denaturation of Ea.**—Approximate concentrations of the reactants for all preparations were 2.4 mg. Ea N per ml. and 0.04 *N* NaOH. The stock 0.10 *N* NaOH used contained less than 0.005 *M* $BaCl_2$. Since the secondary hydrolytic effect of alkali was evident early in the course of denaturation, attempts were made to avoid degradation by determining the time at which analytically detectable amounts of free ammonia first appeared. Fifteen ml. portions of Ea-alkali mixture were placed in a thermostat at 25°. A sample of the original Ea solution, diluted to equal N content, was precipitated with one-third vol. of freshly prepared 20% trichloroacetic acid and the filtrate used as a standard. At intervals of fifteen to twenty minutes 1.5-ml. samples of the reaction mixture were withdrawn, precipitated with trichloroacetic acid, filtered; and compared in matched test-tubes with the same volume of Ea standard filtrate after simultaneous addition of gum ghatti solution and Nessler reagent to both. The time was noted at which ammonia could first be detected, and a larger preparative run was then allowed to proceed for one hour less than this time. In spite of this, other degradative changes were not avoided since a faint odor of hydrogen sulfide could be detected as soon as the reaction mixtures were neutralized.

Salt-free reaction mixtures containing alkali-DnEa were always water-clear, so that aggregation in excess of normal incurred during purification was readily detected by the appearance of opalescence.

3. **Denaturation of Ea in Other Ways.**—Conditions used for the denaturation of Ea by heat or by trichloroacetic acid are given in Table II. The insoluble precipitate (H_2O-DnEa) which separated from a dialyzed, isoelectric solution of Ea during the course of 1 yr. is also included for comparison.

4. **Purification of the Denatured Proteins.**—In the following general purification procedure conditions causing increased opalescence and gel-formation are avoided by carrying out all operations at 0° in low salt concentration and by redissolving the material promptly after each centrifugation.

When the reaction has progressed as far as desired, the chilled reaction mixture is diluted with cold distilled water so that the concentration of sodium chloride will not exceed 0.01 *M* after neutralization. The *p*H is adjusted to the isoelectric range by rapid addition of about 90% of the calculated amount of 0.1 *M* acid or alkali, followed by the remainder in small portions. After each addition of acid or alkali, an aliquot of the mixture is centrifuged. When the supernatant of the test sample is water-clear, the main lot is centrifuged at 0°.[14] The precipitate is then homogenized with a volume of distilled water equal to the volume of the mixture at the initial precipitation, and the minimum amount of 0.1 *M* alkali necessary to dissolve the protein is added with swirling. Reprecipitation and re-solution are repeated as described until samples of two successive supernatants remain clear when boiled or do not react with anti-Ea rabbit serum.

The N concentrations of the fourth and fifth supernatants of the products usually ranged from 0 to 0.003 mg. per ml. Since one or two additional reprecipitations yielded supernatants in the same concentration range, purification was often discontinued when the level of 0.003 mg. N per ml. was reached. The product was finally dissolved at *p*H 7.5 at an approximate concentration of 1% and stored in the ice-box with toluene as preservative. This is referred to as the "aging" process. In no instance was there reason to suspect bacterial contamination. The only soluble material appearing in any supernatant after aging and subsequent isoelectric precipitations was Ea (see below). All supernatants from alkali-DnEa became turbid when warmed to room temperature. Occasionally supernatants from aged acid-DnEa behaved similarly. The small amounts of precipitate which settled over a period of two days did not dissolve when the supernatant was cooled to 0°. No such turbidity was observed when all operations were carried out at room temperature. Therefore, nitrogen analyses on alkali-DnEa supernatants were run on aliquots allowed to warm to room temperature and filtered only after precipitation was complete. The precipitate collected from the supernatants of alkali-DnEa 23, Table II, was purified by isoelectric precipitation and found to show the same quantitative immunological behavior with antiserum to alkali-DnEa as did DnEa 23.

Acid-DnEa which was not allowed to remain in the isoelectric state for more than approximately sixteen hours during purification and was not in contact with more than 0.02 *M* salt (a "normal" product) usually exhibited a bluish opalescence during the first week of aging and was

(11) (a) M. Heidelberger and F. E. Kendall, *J. Exptl. Med.*, **55**, 555 (1932); (b) M. Heidelberger, F. E. Kendall and C. M. SooHoo, *ibid.*, **58**, 137 (1933).

(12) S. D. Henriksen and M. Heidelberger, *J. Exptl. Med.*, **74**, 105 (1941).

(13) G. L. Miller, *J. Biol. Chem.*, **146**, 339 (1942).

(14) Precipitates of alkali-DnEa packed better on centrifugation than did those of acid- or heat-DnEa.

TABLE II

PREPARATION, PURIFICATION, DEGRADATION AND BEHAVIOR ON AGING OF DnEa

	Preparation						Purification						Behavior on aging		
	Ea		Denaturation												
Product DnEa, No.	Lot no.	Wt. N, mg.	pH	Temp., °C.	Time, hr.	Extent, %	Isoelec. pptns., overnight periods	Salt concn. on init. pptn., M	pH of resoln.	Salt concn. on later pptns., M	N in last supnt. before aging,[h] mg./ml.	Description of product	Time aged before reppt., days	N in supnt., mg./ml.	Viscosity
Acid 16[a]	7	1011	1.51	26	49	90.5	4, 1	0.033	3–4	0.008	0	Normal	30	0.038[j]	Decr.
Acid 17	7	644	1.96	25	107	74.5	4	.016	3–4	.002	0.01	Normal	2	.02	Decr.
Acid 21	8	201	1.53	25	49	97	4, 1	.033	3–4	.007	0	Aggr.	20	.016[j]	Decr.
Acid 22	8	201	2.01	25	108	89	5, 2	.01	3–4	.003	0.002	Normal ?	15	.007[j]	Decr.
Acid 32	9	125	1.55	R. t.[e]	29	..	2, 1	<.01	7–8[g]	.001	.002[i]	Normal ?	0.1	.004[i]	Decr.
Acid 33	9	160	1.48	R. t.	37	..	4	<.01	7–8	.001	...	Normal	..	...	Decr.
Acid 33B	..	..	..	...	...	..	([f])	...	...	...	...	Highly aggr.	..	...	...
Alkali 23	8	180	0.041 N[k]	25	2	29.5	3, 1	.01	7–8	.001	0	Normal	6	.025[j]	Const.
Alkali 28	8	413	.041 N[b,k]	25	1.5	28	4, 2	.036	7–8	.01	0.002	Aggr. after 2nd overnt. period	1	.002	Decr.
Alkali 30	6	597	.040 N[c,k]	25	1.1	22	5, 3	.035	7–8	.01	...	Aggr.	..	...	Decr.
Alkali 24[l]	8	404	.040 N[k]	25	3.7	39	5, 3	.01	7–8	<.01	...	Aggr.	..	...	Decr.
Alkali 18[m]	7	561	.048 N[k]	25	43	89	4	.025	7–8	<.01	...	Not aggr.	..	...	Const
Heat 25[n]	8	154	6.8[d]	76	1.1	91	3, 1	<.01	7–8	<.01	...	Highly aggr.	30	0	Decr.
Heat 29[o]	8	165	6.8[b]	80	0.33	97	3, 1	<.01	7–8	<.01	...	Aggr.	..	...	Decr.
Heat 31	9	83	7.3	100	.17	Almost compl.	1	...	7–8	$\ll$0.01	...	Normal	..	...	Const.
TCA-DnEa	By pptn. with 1/4 vol. freshly prepared 20% trichloroacetic acid.						Protein pptble. by TCA and giving strong reaction with anti-Ea rabbit serum found in supnt. obtained after 1st isoelec. pptn. Amount variable; found to be Ea by quantitative immunological analysis. All supnts. TCA-DnEa became turbid on standing.								
H_2O-DnEa	Ppt. which sepd. from sterile salt-free isoelec. solution of Ea during 1 yr.						By isoelec. pptn. Product among least viscous of those studied.								

[a] 0.03% N lost by degradn. [b] 0.004 N NaCl. [c] 0.002 N NaCl. [d] 0.01 M PO_4. [e] Room temp. [f] Part left pptd. for three days. [g] For five minutes. [h] Analyses on dupl. 5-ml. aliquots; values near limit of error of micro-Kjeldahl method. [i] 0.050 mg. N in 25 ml.; supnt. obtained after aging 2.5 hr., 0.104 mg. N per 25 ml. [j] See Table IV. [k] NaOH. [l] 0.2% N lost by degradation. [m] 1.2% N lost. [n] 0.17% N lost. [o] <0.02% N lost.

occasionally gel-like for a few hours. However, if alkali- or heat-DnEa were either opalescent or gel-like, it was a certain indication of excessive aggregation due to one or both of the factors mentioned above.

5. Analysis of Initial Supernatants for Non-protein Nitrogen.—It has been reported that nitrogen and hydrogen sulfide are split from Ea by the action of various denaturants.[15,16,17,18] In the present studies, therefore, the amount of NPN formed was determined by micro-Kjeldahl analyses on simultaneously prepared trichloroacetic acid filtrates of the initial supernatants of the products and Ea solutions of equal N content. Such filtrates from Ea solutions containing up to about 1 mg. N per ml. showed not more than 0.02 ± 0.006 mg. N per 30 ml. of filtrate. From this and from the figures on DnEa supernatants given in Table II, it is concluded that the amounts of N split from the products in which no degradation could be detected by other tests as well was not greater than 0.1% of the total and was probably often less than this. Several alkali-DnEa preparations, deliberately treated as drastically as by earlier workers, showed N losses of 1 to 2%.

6. The pH Range of Complete Precipitation of DnEa by 0.1 M Acetate Buffer.—At a concentration of about 1 mg. N per ml., acid-DnEa is completely precipitated (within 1%) between pH 4.7 and 5.5. The range of complete precipitation for heat-DnEa is about the same, while that for alkali-DnEa is between narrower limits.

Data on the preparation, purification, degradation and behavior on aging of the products are summarized in Table II. The results of quantitative immunological analyses on the supernatants of acid-DnEa described in column 17 of Table II are given in Table III.

(15) H. Wu and D. Y. Wu, *J. Biochem (Japan)*, **4**, 345 (1924).

(16) M. Sörensen and S. P. L. Sörensen, *Compt. rend. trav. lab. Carlsb.*, **15**, Art. 9 (1925).

(17) B. M. Hendrix and J. Dennis, *J. Biol. Chem.*, **126**, 315 (1938); *Arch. Biochem.*, **2**, 371 (1943).

(18) R. C. Warner and R. K. Cannan, *J. Biol. Chem.*, **142**, 725 (1942).

TABLE III

TOTAL N PRECIPITATED FROM 1.0 ML. ANTI-Ea RABBIT SERUM 748, 1:1, BY SUPERNATANTS OF ACID-DnEa PREPARATIONS, AND BY Ea 7[a]

Solution added	N added, mg.	Total N pptd., mg.	Approx. % EaN indicated, calcd. from calibration curve[10,11]
Supnt. 5 DnEa 16	0.038	0.484	100
DnEa 16[b]	.038	.036	
Ea 7	.038	.495	
Supnt. 5 DnEa 21	.024	.275	80
Ea 7	.024	.340	
Supnt. 6 DnEa 22	.017	.222	90
Ea 7	.017	.250	
Supnt. 4 DnEa 23	.038	.244	50
Ea 7	.038	.495	

[a] Serum 748 was calibrated with Ea 7. The values of total N pptd. by Ea 7 are derived from the calibration curve. [b] A dilution of a portion of DnEa 16 which had not been given a fifth precipitation. About 2% of its N content was Ea N (see Table II and Discussion).

Results and Discussion

Generally, the "normal" appearance and behavior of acid-, alkali-, and heat-DnEa were lost if, during purification, they were exposed to salt in excess of approximately 0.02 N or were allowed to stand in isoelectric suspension for more than about sixteen hours (*i. e.*, overnight). The physi-

cal meaning of the resulting changes is discussed in the following paper. Although some lots of acid-DnEa were not perceptibly aggregated by approximately 0.035 *M* NaCl, others were. The various preparations of alkali-DnEa were somewhat less sensitive to salt. Since there is no difficulty in keeping the salt concentration low, it seems best to specify 0.01 *M* sodium chloride as the maximum concentration of electrolyte permissible for acid- or alkali-DnEa when these are desired in the "normal" state.

The presence of only 0.004 *M* salt (DnEa 29, Table II) caused considerable aggregation during denaturation by heat.

The prevention of loss of nitrogen during denaturation by alkali required considerable care. Products which were deliberately degraded became less easily altered by salt or by long periods in isoelectric suspension. The carefully prepared alkali- and acid-DnEa preparations described herein are probably the first from which the adjective "degraded" might legitimately be omitted.

When preparations of acid-DnEa were purified by re-solution at *p*H 3–4 and were then aged for varying lengths of time at *p*H 7–8, subsequent isoelectric precipitation left Ea amounting to 1% to 2% of the total protein in the supernatants (Table II, column 17 and Table III). The Ea was not found after subsequent aging periods, provided the DnEa had aged for two to three days in the first instance. The appearance of Ea in the supernatant might have been due to the complete reversion of a very small fraction of the acid-DnEa to Ea or to the slow dissociation at *p*H 7–8 of a complex of Ea and DnEa. That Ea appears in measurable amounts in even a few hours is illustrated by the data on DnEa 32 (Table II). After the second precipitation this was allowed to remain in solution (or aged) at *p*H 7–8 for two and a half hours, and was then reprecipitated. The third supernatant contained twice as much nitrogen as the second. All acid-DnEa solutions should therefore be tested for Ea at least once after they have aged, preferably for several days.

In this connection, the behavior of trichloroacetic acid-DnEa (TCA-DnEa) is of interest. When Ea was completely precipitated by trichloroacetic acid and then redissolved at *p*H 7–8 and isoelectrically reprecipitated, 4–10% of the total protein was always present in the supernatant. This protein was found to be Ea by quantitative immunochemical analysis.

With one exception, Ea was not found in aged solutions of the lots of alkali- or heat-DnEa studied. These were purified by re-solution at *p*H 7–8, with short intervals only, between precipitations. After aging, the solutions were tested periodically for Ea after isoelectric precipitation by quantitative analyses of the supernatants by chemical and immunological methods. The one exception was alkali-DnEa 23 (Tables II and III) in which a small fraction was found having the solubility of Ea but not its typical immunological behavior.

Summary

1. The controlled preparation of various types of relatively undegraded DnEa has been described and conditions have been given under which these very sensitive types of DnEa may be purified by isoelectric precipitation and re-solution without increase in apparent aggregation.

2. The nitrogen split from acid-DnEa and from heat-DnEa produced by rapid denaturation was far less than 0.1% of the total nitrogen denatured. Nitrogen lost by the alkali-DnEa products varied from less than 0.1% to over 1%, depending on the severity of the treatment with alkali. Sensitivity to standing in the isoelectric state or to salt decreased with the extent of degradation.

3. Various preparations of Ea were denatured by acid at widely different rates.

4. "Normal" lots of acid-DnEa lost their gel-like appearance, when present, in a few hours and their opalescence in a few days. "Normal" lots of heat- or alkali-DnEa were water-clear and did not change in appearance on aging. Severely aggregated products of all types retained their gel structure for days and, although their opalescence decreased on aging, it seldom disappeared entirely. Duration of both phenomena depended on the degree of aggregation.

5. After supposedly purified acid-DnEa had aged at *p*H 7–8 and 0–5°, Ea was found in the solutions. Once this Ea was removed no more appeared. No Ea appeared in purified aged solutions of heat-DnEa or (with one curious exception) those of alkali-DnEa.

NEW YORK, N. Y. RECEIVED DECEMBER 9, 1944