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Denatured Egg Albumin. II. Viscosities, Particle Weights and Electrophoretic Mobilities of Crystalline Egg Albumin Denatured in Various Ways[1]

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In the preceding paper[1a] it was noted that the initial opalescence of neutral solutions of acid-DnEa and of the aggregated lots of alkali- or heat-DnEa gradually disappeared on standing at 0–5° (*i. e.*, aging). If gel structure and opalescence were exhibited by heat- or alkali-DnEa, it required months for the solutions to clear. On the other hand, solutions of "normal"[2] acid-DnEa usually remained opalescent for only a few days.

The present study of the viscosities and particle weights of the preparations[1a] was undertaken in order to follow some of the changes, apparently hitherto unobserved, which occurred on aging. The electrophoretic mobilities of a number of the preparations were also determined.

Experimental

1. **Viscosity.**—The viscosities of most of the lots of DnEa were determined as soon as possible after isolation and at varying intervals thereafter until the values ceased to change. A 4–5 ml. aliquot of DnEa solution was dialyzed at 0–5° for two to four days, in the presence of toluene, against two changes daily of 0.02 *M* phosphate buffer at *p*H 7.95 and ionic strength 0.06. Because Polson[3] had shown that 0.01 *M* Na_2HPO_4 was ample to suppress the electroviscous effect in the case of Ea it was decided to use twice this concentration of buffer for most of the runs as a compromise between a possibly greater electroviscous effect of DnEa and its known salt-sensitivity. Viscosity determinations were made with 2-ml. portions of dialyzed solution in an Ostwald viscometer at 25 ± 0.05°. At least two concentrations of protein were used, the weaker being obtained by dilution of the original dialyzed solution with buffer. The nitrogen content of one of these solutions was determined by micro-Kjeldahl analysis. Usually the nitrogen concentrations were so chosen that the time of outflow was at least 120 sec. as compared with about 109 sec. for the buffer alone. The usual average deviation for 4–5 runs was ±0.1 sec. No corrections were made for the small differences in density between the protein solutions and the buffer, as the over-all accuracy was limited to 1–2% by the method of analyzing for nitrogen.

After it was found that the relative fluidity of "normal" acid-DnEa was a linear function of the concentration[4] up to at least 2.0 mg. N per ml., all results were graphically expressed in this manner since only two determinations establish a line. In addition, the fluidity, or its reciprocal, the viscosity, could be readily interpolated for any other concentration within the linear range. In Figs. 1–6 concentration in mg. N per ml. was plotted against the relative fluidity. As the nitrogen concentration is proportional to the volume fraction (vol. fraction = vol. protein/vol. solution = wt. protein × partial sp. vol./vol. solution), this is the procedure of choice until exact data are obtained on the partial specific volumes of various types of DnEa and their nitrogen content.[5] In calculating the axial ratios of the products from the viscosities it was assumed, as an approximation, that the partial specific volumes and the nitrogen content were the same as for native egg albumin (Ea).

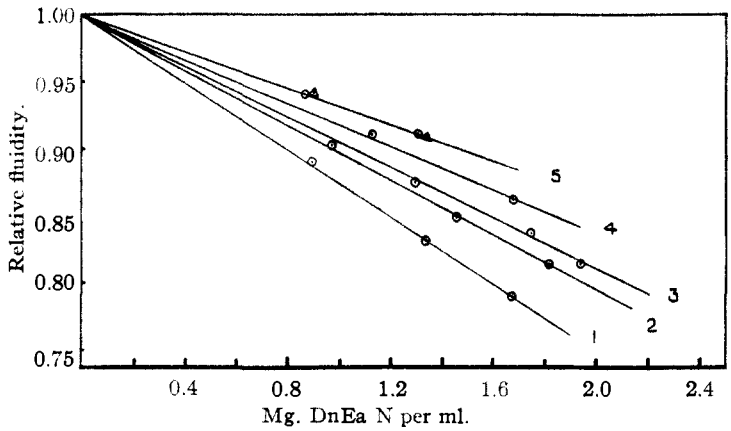

Fig. 1.—Increase in relative fluidity of "normal" acid-DnEa 17 on aging: line 1, after two days; line 2, after seven days; line 3, before and after two days in isoelectric suspension after aging about four weeks; line 4, after aging two months; line 5, aged for six (circles) and ten (triangles) months. Acid-DnEa 16 behaved similarly.

2. **Electrophoresis.**—Mobilities were determined in the Tiselius apparatus[6] using the scanning method of Longsworth.[7] The medium was 0.02 *M* phosphate buffer at *p*H 7.5. The protein concentration was approximately 0.5%. Conductivities of protein solution and buffer were determined after dialysis for 24 hours. The electric field strength was 8.9 volts per cm. Four scanning exposures were made for each product at evenly spaced intervals. The averaged mobility values are listed in Table I. The patterns shown in Fig. 7 were obtained after passage of the current for one hour.

3. **Diffusion.**—Measurements were carried out in the Tiselius apparatus at 1, 2, or 5°, using the Philpot-Svensson cylindrical lens[8] or the scanning method of Longsworth. The protein solutions were dialyzed as described in the preceding paragraph. The boundaries were formed and brought into view in the usual manner. The diffusion con-

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(1a) C. F. C. MacPherson and M. Heidelberger, THIS JOURNAL, **67**, 574 (1945).

(2) *I. e.*, preparations exposed to minimal aggregative influences during isolation and storage.[1a]

(3) A. Polson, *Kolloid Z.*, **88**, 51 (1939), esp. Tables IV and VI.

(4) *Cf.* H. P. Treffers, THIS JOURNAL, **62**, 1405 (1940), for fluidity studies on other proteins.

(5) B. M. Hendrix and J. Dennis, *J. Biol. Chem.*, **126**, 315 (1938).

(6) A. Tiselius, *Trans. Faraday Soc.*, **33**, 524 (1937).

(7) L. G. Longsworth, THIS JOURNAL, **61**, 529 (1939).

(8) (a) J. St. L. Philpot, *Nature*, **141**, 283 (1938); (b) H. Svensson, *Kolloid Z.*, **87**, 181 (1939).

TABLE I

MOBILITIES OF DnEa AND Ea IN 0.02 *M* Na_2HPO_4-NaH_2PO_4 BUFFER AT *p*H 7.5

Preparation	Mobilities × 10^5 sq. cm./volt-sec. Ascending	Descending	Remarks
Ea 6	9.9	9.2	Main component
	8.4	8.2	Satellite
Acid-DnEa 22	9.4	8.8	Homogeneous
Acid-DnEa 16	8.8	9.2	Homogeneous
Heat-DnEa 29	9.4	9.1	Homogeneous
Alkali-DnEa 30	6.9	6.7	Main component
	5.0	4.8	5% of total

stants listed in Table II, column 4, are averages of values obtained from the two arms of the U-tube and were calculated by the maximum height method.[9] The patterns obtained were somewhat skewed, except in the final runs with DnEa 17 and 32, but when their maximum heights were plotted as ordinates against the reciprocals of the square roots of the times at which the patterns were taken, the maxima fell either on or very near to a straight line passing through the origin.

4. Sedimentation.—Sedimentation runs were made in an air-driven ultracentrifuge[10] kindly placed at our disposal by Dr. Aura E. Severinghaus, of the Department of Anatomy. Unless otherwise stated, 0.5 *M* phosphate buffer at *p*H 7.9 was added to the salt-free DnEa solution immediately before it was placed in the cell, to make a final electrolyte concentration of 0.1 *M*. This was done in the hope of reducing salt aggregation to a minimum, since it had been shown that 0.1 *M* phosphate caused a considerable increase in the viscosity and opalescence of acid-DnEa after dialysis for four days at 0–5°. However, some opalescence developed within an hour in every case. For this reason, comparative runs were made with DnEa 21, the most salt-serisitive of the acid preparations, in 0.02 *M*, 0.05 *M*, and 0.1 *M* phosphate buffers to determine if the additional aggregation indicated by the gradually developing opalescence was discernible in the ultracentrifuge. The sedimentation constants found in 0.02 and 0.1 *M* buffer during the thirty-minute period of observation were practically the same, namely, 11.8 and 12.0. Moreover, the areas of the patterns in the runs in 0.02 and 0.05 *M* buffer, the only ones measured, did not decrease significantly, indicating that further aggregation had not ensued. To determine whether or not a fraction of the material was instantly aggregated by the salt and precipitated from solution before exposures were made, a 1% solution of DnEa 21, freshly made in 0.1 *M* phosphate buffer, was studied as follows: The centrifuge was accelerated to 22,000 r. p. m. and then allowed to stop. No precipitate could be seen when the cell contents were examined with a lens. The solution was then drawn up in a syringe and sprayed back into the cell so that the jet hit the bottom, dislodging a few thread-like particles which could have contained no more than a few tenths % of the total protein. These experiments, then, indicate that the sedimentation constants found are those of particles unaggregated during the brief period of exposure by the concentrations of salt in which the runs were made (Table II).

TABLE II

SEDIMENTATION AND DIFFUSION CONSTANTS OF DnEa

Product	Final η rel. of 0.65% protein solution	Diffusion: Protein concn., %	Diffusion: $D^{20}_{H_2O}$ × 10^7	Sedimentation: Protein concn., %	Sedimentation: Electrolyte concn., *M*	S_{20} Svedbergs
Acid-DnEa 16	1.078	0.5	2.4	0.7	0.1	9.1
Acid-DnEa 17	1.078			.8	.1	9.1
Acid-DnEa 17[a]		.6	2.5			
Acid-DnEa 21	1.117	.9	1.4	1.0	.1	12.0
Acid-DnEa 21	1.117	.4	1.7	1.0	.05	11.9
Acid-DnEa 21	1.117			1.0	.02	11.8
Acid-DnEa 32[b]	(1.12)	1.0	(0.6)	1.0	.02	25.0
Acid-DnEa 32[c]	(1.10)	1.0	2.0	1.0	.02	13.3
Acid-DnEa 32[d]		0.8	2.6	0.6	.02	11.3
Heat-DnEa 29	1.112	.9	1.8	.7	.1	15.9
Heat-DnEa 31	1.042	1.2	4.2	.4	.02	9.0
Alkali-DnEa 24	1.071	0.8	2.3	1.0	.1	8.2
Alkali-DnEa 30	1.203	.5	1.8	1.0	.1	10.1

[a] After four years. [b] Solution aged two to three days. [c] Solution aged three weeks. [d] After approximately two years. Values in parentheses are uncertain.

During the first half of all sedimentation runs only a single boundary was present. After this time (thirty minutes) the boundaries gradually became so diffuse that runs were terminated at the end of an hour.

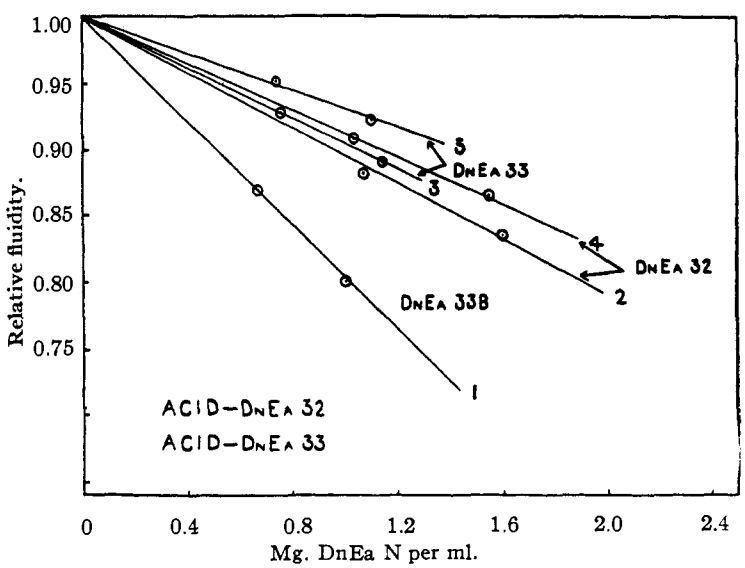

Fig. 2.—Line 2 is relative fluidity of "normal" acid-DnEa 32 before aging (no. 32 had been allowed to remain in isoelectric suspension overnight during purification). Line 4 is after aging for about a month. Line 3 is relative fluidity of "normal" acid-DnEa 33 before aging (no. 33 had been allowed to remain in isoelectric suspension only a few hours during purification); line 5, aged for about two months. Line 1 is relative fluidity of aggregated acid-DnEa 33B (portion left in isoelectric suspension for three days during purification), after aging about two months.

5. Additional Data on Heat-DnEa 31 and Acid-DnEa 32.—About two years after the other figures in Table II were obtained, diffusion runs were made at 1° on 0.8 and 0.4% solutions of DnEa 32. At the lower concentration, a slight drift in the values was noted, but the average of the last three readings was taken. D_{20} was calculated to be 2.6 and 2.5 at the two concentrations. Both values were appreciably higher than those given by the same preparation after aging only three weeks. New sedimentation runs were also made, at 1% protein concentration in 0.02 *M* phosphate buffer, at 0.6% concentration in the same buffer, and at 0.4% concentration in 0.007 *M*

(9) O. Lamm and A. Polson, *Biochem. J.*, **30**, 528 (1936).

(10) J. A. Chiles, Jr., and A. E. Severinghaus, *J. Exptl. Med.*, **68**, 1 (1938); *Rev. Scientific Inst.*, **11**, 71 (1940).

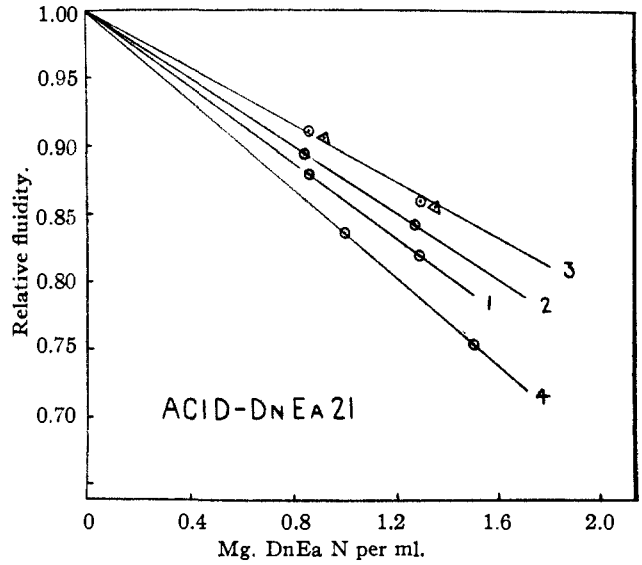

Fig. 3.—Line 1 is relative fluidity of aggregated acid-DnEa 21 before aging; line 2, after three weeks; line 3, aged for about eight months (circles) and eleven months (triangles). Line 4, after about eleven months, then exposed to 37°, 0.02 *M* phosphate buffer, for ten days.

phosphate and 0.04 *M* KCl. The values for S_{20} were 10.7, 11.3, and 11.4, considerably lower than given by the three-weeks old solution. There was little noticeable drift with decrease in concentration.

Similarly, diffusion runs on heat-DnEa 31 in 1.2 and 0.6% solution gave a value of 4.2×10^{-7} for D_{20} in both instances. Sedimentation runs in 0.02 *M* phosphate buffer at concentrations of 1.2 and 0.4% gave values of 8.7 and 9.0 for S_{20}, respectively. The latter was used for calculation of the particle weight (Table III).

Results and Discussion

1. Fluidity and Viscosity.—Early in the aging process, the fluidities of some "normal" acid-DnEa preparations could not be measured at nitrogen concentrations much higher than 2 mg. per ml. because the time of outflow increased each time a run was repeated. For example, after DnEa 16 had aged for four days, it was run at a concentration of 4.36 mg. N per ml. The time of outflow increased by about 0.5 sec. with each run. On dilution to 2.18 mg. N per ml. the time of outflow was constant. This behavior was also shown by a solution of DnEa 17 (Fig. 1), aged four days, containing 2.49 mg. N per ml., while one month later the time of outflow was constant. The highly aggregated heat-DnEa 25 could not be run at concentrations exceeding 0.5 mg. N per ml., even after several months of aging. Below these nitrogen concentrations, which differed with the extent of aggregation of the products, the fluidity–concentration relationship was linear.

It is possible that electroviscous effects due to a concentration of ionized groupings on the large aggregates initially present[11] were partly responsible for these irregularities of flow and that such effects decreased as the acid-DnEa aggregates diminished in size on aging. However, the ultracentrifugal data, showing that no immediate change in sedimentation occurred with increase in salt concentration, indicate that once the viscosity became constant at any rate, the viscosity values in 0.02 *M* buffer contained no component due primarily to the electric charge on the protein particles.

The fluidities of all lots of acid-DnEa studied increased during aging (*i. e.*, the viscosities decreased), the greatest change taking place during the first weeks (Figs. 1–3).

Precipitation at the isoelectric point and storage at 0–5° for about sixteen hours (*i. e.*, overnight) during purification did not decrease the initial fluidity of acid-DnEa markedly. For instance, lines 2 and 3, Fig. 2, denote the initial fluidities of DnEa 32 and DnEa 33, analogous preparations except for the length of time they were in isoelectric suspension. However, when a portion of No. 33 was left in the isoelectric state for as long as three days, it formed a very opalescent, highly salt-sensitive, neutral solution of low fluidity (DnEa 33 B, Fig. 2). After aging had progressed for one to two months, two days in the isoelectric state had no measurable effect on the fluidity (line 3, Fig. 1). After acid-DnEa 16, also, had aged one month, its fluidity and appearance were not perceptibly changed after it had been precipitated for as long as five days.

The aggregating effect of salt in excess of 0.02 *M* was exerted regardless of the age of the product and generally was more pronounced the lower the fluidity. This effect with aged acid-DnEa 16 is shown in Fig. 4. Both the decrease in fluidity and the increase in opalescence of the solutions

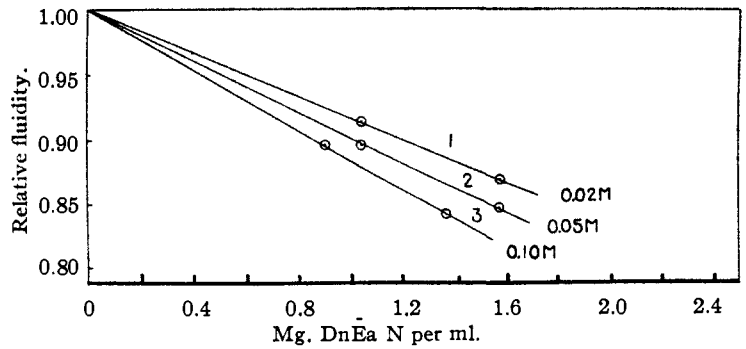

Fig. 4.—Relative fluidities of "normal" acid-DnEa 16 dialyzed four days at 0–5° against 0.10, 0.05 and 0.02 *M* phosphate buffers of *p*H 7.9. Preparation had aged for about five months.

(11) *Cf.*, for example, M. Heidelberger and F. E. Kendall, *J. Biol. Chem.*, **95**, 127 (1932).

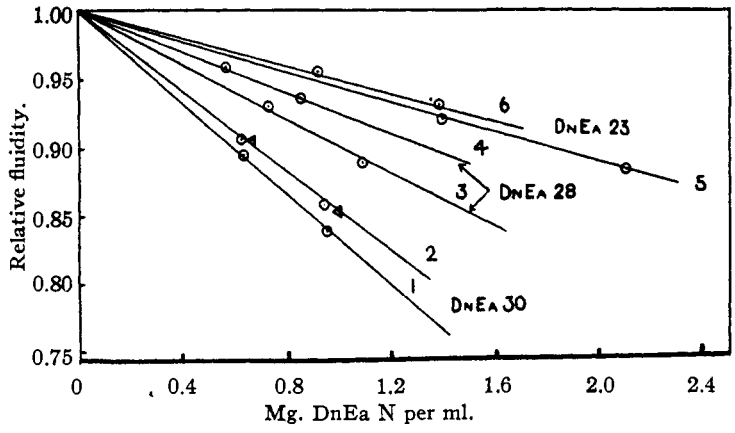

Fig. 5.—Relative fluidities of three undegraded lots of alkali-DnEa (30, 28 and 23): line 1, aggregated DnEa 30 after aging one day; line 2, after about one month (circles) and three months (triangles); line 3, aggregated DnEa 28 after aging for one day; line 4, after six weeks; line 6, "normal" DnEa 23 before aging; line 5, after aging for one month.

were in proportion to the concentration of salt. Since the original solution of DnEa 16 contained slightly less than 0.01 *M* salt during the first month of aging when the greatest increase in fluidity occurred, this concentration of salt was shown to be non-aggregating and was therefore adopted as the maximum to be used in purifying all types of DnEa.[1a]

In addition to salt in excess of 0.02 *M* or prolonged periods in the isoelectric state before aging, exposure of DnEa solutions to room temperature or above also led to excessive aggregation. It was noted repeatedly that aggregated preparations of any type, the solutions of which were still rather opalescent, or "normal" acid-DnEa lots which had not aged sufficiently for their fluidities to reach 0.92 or more at a concentration of 1 mg. N per ml., always became more opalescent and sometimes precipitated when left at 37°. The opposite effect of lowering the temperature was reported by Mirsky,[12] who noted that this reduced the size of aggregates formed when water was added to a solution of DnEa in 9.5 *M* urea. The decrease in fluidity after exposure to 37° was measured for acid-DnEa 21, a somewhat aggregated preparation, after its fluidity had been constant for three months. One aliquot was dialyzed for ten days at 37° against 0.02 *M* phosphate buffer in the presence of toluene (line 4, Fig. 3) and another was treated similarly, but at 0–5° (triangles, line 3, Fig. 3). The large decrease in the fluidity of the aliquot exposed to 37° is clearly shown.

The data in Figs. 1–3 indicate variation in both the initial fluidities of "normal" lots of acid-DnEa and the times required for the fluidities to become constant. Satisfactory explanations for these differences are not available. In addition to small variations in the salt concentrations to which the products were exposed or the length of time they were allowed to stand in the isoelectric state before aging, other possible factors are the lot of Ea used, the *p*H of denaturation, and the *p*H at which re-solution was effected during purification.

The fluidity of alkali-DnEa which was not exposed to excess salt during purification or allowed to remain in isoelectric suspension for much more than sixteen hours (overnight) was relatively high and did not change appreciably on aging. The fluidities of the aggregated products decreased somewhat on aging, although not nearly to the extent of acid-DnEa (Fig. 5). The fluidity of the deliberately degraded DnEa 18 which had lost 1.2% of its N during denaturation was little different from that of the undegraded alkali-DnEa 23.

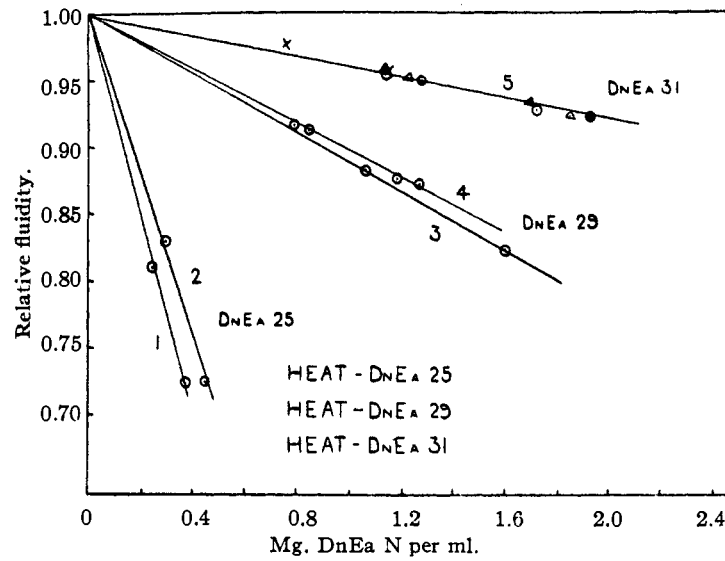

Fig. 6.—Lines 1 and 2 describe the relative fluidities of highly aggregated DnEa 25 after aging for about one and five months; line 3, aggregated DnEa 29 after aging for six days; line 4, after one and four months; line 5, "normal" DnEa 31 in 0.02 *M* NaCl (crosses) and 0.02 *M* phosphate buffer (half filled circles) before purification by iso-electric precipitation; in 0.02 *M* NaCl (circles) and 0.02 *M* phosphate buffer (open triangles) after one isoelectric precipitation; after aging for three months (solid triangles).

Allowing undegraded alkali-DnEa to remain

(12) A. E. Mirsky, *Cold Spring Harbor Symposia on Quant. Biol.*, 9, 228 (1941).

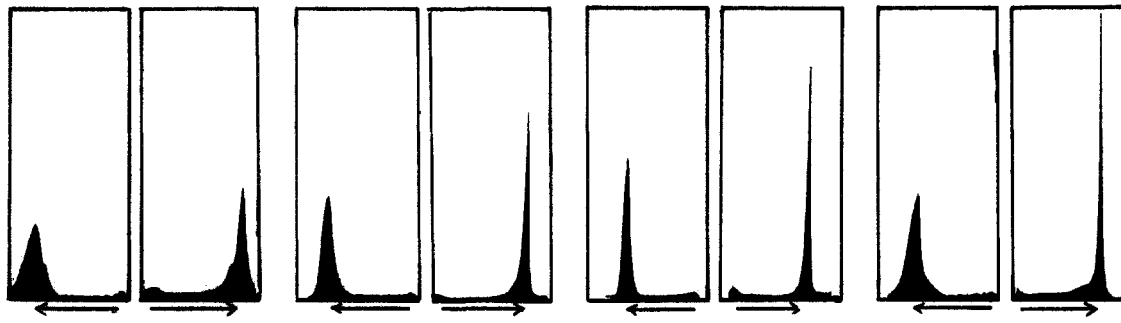

Fig. 7.—Electrophoresis patterns of Ea, acid-DnEa 22, heat-DnEa 29 and alkali-DnEa 30 in 0.02 *M* Na_2HPO_4–NaH_2PO_4 buffer at *p*H 7.5 after one hour.

for more than one overnight period in the isoelectric state during isolation led to highly opalescent, salt-sensitive products of low fluidity. The relative positions of the initial fluidity–concentration lines (Fig. 5) of the undegraded lots 23, 28 and 30 correlate in a rather striking manner with the lengths of time they were left in the isoelectric state (1, 2 and 3 overnight periods, respectively).

Two degraded lots of alkali-DnEa were studied. DnEa 24 had lost 0.2% N and DnEa 18 lost 1.2% N in the prolonged treatment with alkali to which they had been subjected (Table II, paper I).[1a] Although DnEa 24 was also kept in isoelectric suspension for 3 overnight periods during purification, it formed a solution which was only slightly opalescent. Comparison of its initial fluidity–concentration line, which lay between lines 3 and 4 of Fig. 5, with that of alkali-DnEa 30, an undegraded preparation which had also stood for 3 overnight periods during purification, illustrates how a small degree of degradation greatly diminishes the tendency to aggregate. Several other degraded preparations showed similar behavior in proportion to the degree to which hydrolytic cleavage of N had occurred.

The fluidity–concentration relationships of three lots of heat-DnEa are shown in Fig. 6. DnEa 25 (highly aggregated) was denatured in the presence of 0.01 *M* phosphate at *p*H 6.8, DnEa 29 (aggregated) was denatured in about 0.004 *M* sodium chloride at *p*H 6.8, and DnEa 31 (normal) in salt-free solution at *p*H 7.3. These results show the extraordinary aggregating effect of minute amounts of salt during denaturation by heat, as has been noted by Rothen[13] and many others. In contrast, the presence of 0.004 *M* sodium chloride during alkaline denaturation, as in the preparation of DnEa 28, caused no comparable aggregation, the reaction mixture remaining water-clear.

Immediately after heat-DnEa 31 was prepared, aliquots of the cooled solution were dialyzed against 0.02 *M* phosphate and 0.02 *M* sodium chloride. The remainder of the solution was isoelectrically precipitated, centrifuged at room temperature, and the precipitate was then redissolved in the supernatant by addition of the minimum amount of alkali. Aliquots of this solution were dialyzed against the same solvents. As shown in Fig. 6, the fluidities of the precipitated and non-precipitated portions were identical in both solvents, demonstrating that isoelectric precipitation does not decrease the fluidity of this type of DnEa even when carried out shortly after the denaturation.

The fluidity of DnEa 31 was slightly higher than that given by Bull[14] for Ea denatured by heat in salt-free solution.

H_2O-DnEa was the precipitate which separated from a sterile, salt-free, isoelectric solution of Ea in the course of a year. Its fluidity–concentration line almost coincided with that for DnEa 31, the least viscous of all the lots of DnEa studied. In view of the effect of long standing in the isoelectric state on acid- or alkali-DnEa, the comparatively high fluidity of this product was surprising.

No experiments were done to ascertain whether the various products obeyed the Hagen–Poiseuille law. However, Bull[14] found this law to be followed by a sample of heat DnEa comparable to DnEa 31.

Axial ratios of the various products which have been described in this paper were calculated from the viscosity data and are listed in Table III. The equations and data of Simha[15,16] were used, as these appear to be the most acceptable.[3,17] Axial ratios calculated from viscosity data could scarcely indicate the extent of "unrolling" or "distension" of the native Ea molecule induced by certain denaturants[14,18] unless it were known that the protein was in the monomolecular state in the solvent used for the viscosity studies. DnEa has been shown to be dispersed to this extent only in the presence of urea, while the diffusion and sedimentation data point clearly toward extensive aggregation even in water containing minimal amounts of acid, alkali, or salt.

(13) A. Rothen, *Annals N. Y. Acad Sci.*, **43**, 229 (1942).

(14) H. B. Bull, *J. Biol. Chem.*, **138**, 39 (1940).

(15) R. Simha, *J. Phys. Chem.*, **44**, 25 (1940).

(16) J. W. Mehl, J. Oncley and R. Simha, *Science*, **92**, 132 (1940).

(17) H. B. Bull, *Advances in Enzymology*, **1**, 1 (1941).

(18) W. J. Loughlin, *Biochem. J.*, **26**, 1557 (1932).

TABLE III

CALCULATED AXIAL RATIOS, ASYMMETRY FACTORS, AND PARTICLE WEIGHTS[a] OF DnEa

Product	Period aged	Viscosity: $\eta_{sp}{}^{d}$ $\varphi_c \rightarrow 0$	Viscosity: Rods $1/\rho$	Viscosity: Rods f/f_0	Viscosity: Rods Particle weight	Viscosity: Disks ρ	Viscosity: Disks f/f_0	Viscosity: Disks Particle weight	Sedimentation and diffusion f/f_0	Sedimentation and diffusion Particle weight
Acid-DnEa 16(n)[b]	4 dys.	22.2	13.9			31.0				
Acid-DnEa 16(n)	11 mos.[c]	14.0	10.2	1.55	600,000	18.8	1.7	500,000	1.85	400,000
Acid-DnEa 17(n)	2 dys.	27.0	15.6			37.3				
Acid-DnEa 17(n)	6 mos.[c]	14.0	10.2			18.8				
Acid-DnEa 21(a)	0 dys.	29.7	16.7			42.0				
Acid-DnEa 21(a)	8 mos.[c]	22.0	13.8	1.7	2,100,000	30.7	2.0	1,300,000	2.4	800,000
Acid-DnEa 32(n)	2–3 dys.								3.2	3,800,000
Acid-DnEa 32(n)	3 wks.	18.3	12.2	1.65	1,100,000	25.2	1.9	700,000	1.9	700,000
Acid-DnEa 32(n)	2 yrs.								1.7	400,000
Heat-DnEa 29(a)	1 mo.[c]	21.4	13.6	1.7	1,200,000	29.8	2.0	800,000	1.9	900,000
Heat-DnEa 31(n)	0 dys.[c]	8.6	7.0			10.8				
Heat-DnEa 31	2 yrs.								1.3	200,000
Alk-DnEa 23(n)	1 dy.	9.9	7.9			12.7				
Alk-DnEa 24(a)	3 mos.[c]	15.0	10.7	1.6	600,000	20.2	1.8	500,000	2.0	300,000
Alk-DnEa 30(a)	1 mo.[c]	29.7	16.7	1.9	900,000	42.0	2.2	500,000	2.2	500,000
Ea (for comp.)		5.7[15]	5.0[15]			6.7[15]			1.2[24]	40,000[24]

[a] To nearest hundred thousand. [b] (n) signifies a "normal" product, (a) an aggregated one. [c] Fluidity constant. [d] For symbol ν see ref. 15.

The viscosity data alone permit no conclusions as to the mechanism of the decrease in viscosity undergone by DnEa on aging, since either a change in shape brought about by a condensation or rolling up of the polypeptide chains or fabrics[19] of the individual molecules making up the aggregate, or splitting of the aggregates could bring about the same changes in axial ratios.

2. Electrophoresis

The mobilities at *p*H 7.5 of two lots of fully aged acid-DnEa and one aggregated, aged, heat-DnEa were essentially the same as that of the main component of egg albumin.[7,20,21] The preparations were electrophoretically homogeneous (Table I and Fig. 7). These results supplement observations[21] that the mobilities of Ea completely denatured by heat or acid were but slightly lower than those of the native form at *p*H 6.8 and 10.3. In contrast, the mobility of undegraded, aggregated alkali-DnEa 30 was considerably below that of either component of Ea, in agreement with Longsworth's finding[22] that in a solution of Ea undergoing alkali-denaturation at *p*H 12.8 the mobility of the component that increased with time was less than that of Ea.

3. Diffusion and Sedimentation

In 1930 Nichols[23] found that, after three hours in 0.1 *N* HCl, the sedimentation constant of egg albumin (Ea) increased roughly four times. Rothen[13] denatured Ea by heat in the absence of salt and found the sedimentation constant to be double that of Ea. Rothen and Mirsky[13] confirmed earlier evidence that in the presence of urea the molecular weight of Ea was unchanged but believed that the molecule was completely unfolded. Removal of the urea by dialysis resulted in aggregation. From these studies it appeared probable that all types of denatured egg albumin would be found to exist as aggregates in water containing just enough alkali for solution.

The changes which occurred during the initial aging period of a "normal" acid-DnEa (32) were followed by ultracentrifugal and diffusion measurements to ascertain if the decrease in opalescence and viscosity previously described were due to changes in particle weight or only to changes in shape. At the time the sedimentation and diffusion constants were measured on the other products (with the possible exception of DnEa 30) these had aged until their viscosities were substantially constant.

The diffusion constants of representative lots of "normal" and aggregated acid-, heat- and alkali-DnEa were determined and are listed in Table II, column 4. All but two of the products were run at only a single concentration. In one of the exceptions, the highly aggregated DnEa 21, the diffusion constant was found to be larger at the lower concentration, 0.4%. Lamm and Polson[9] and Polson[3] have observed a few other proteins to behave similarly. In the case of the other product, DnEa 32, after two years of aging, changes in D on dilution from 0.8 to 0.4% were scarcely outside experimental error, but the values at the lower concentration showed a continuous drift downward, making the result uncertain.

Except in the final DnEa 17 run and the DnEa 32 run at 0.8% concentration, most of the diffu-

(19) D. Wrinch, *Cold Spr. Harbor Sympos. Quant. Biol.*, **6**, 122 (1938).

(20) K. Landsteiner, L. G. Longsworth and J. van der Scheer, *Science*, **88**, 83 (1938).

(21) L. G. Longsworth, R. K. Cannan and D. H. MacInnes, THIS JOURNAL, **62**, 2580 (1940).

(22) L. G. Longsworth, *Annals N. Y. Acad. Sci.*, **41**, 267 (1941).

(23) (a) J. B. Nichols, THIS JOURNAL, **52**, 5176 (1930); (b) B. Sjögren and T. Svedberg, *ibid.*, **52**, 5187 (1930).

sion curves were slightly skewed, so that ideality of diffusion can scarcely be claimed. For this reason the particle weights calculated with the aid of these values can be little more than rough approximations and are therefore given only to the nearest hundred thousand.

While the various lots of DnEa showed only one boundary in the electrophoresis cell and in the ultracentrifuge, the rapidity with which spreading of the boundary set in during sedimentation suggested that the preparations were mixtures of polymers not greatly different in particle size.

Of the six stabilized proteins studied in the ultracentrifuge, acid-DnEa 16 and 17 (S = 9.1) were not obviously aggregated by the process of isolation, while DnEa 21 (S = 12) was. As for alkali-DnEa lots 30 and 24 (S = 10.1 and 8.2, resp.) the former was apparently highly aggregated during isolation (S was determined after aging) and the latter only slightly. Heat-DnEa 29 (S = 15.9) was aggregated during its production in the presence of 0.004 M NaCl, while heat-DnEa 31, prepared without added electrolyte, showed S = 9. Heat-DnEa 31, with its low dissymmetry constant as well as low S, had the smallest particle weight, 200,000, encountered in the course of the study.

Particle weights were calculated according to the Svedberg formula,[24] using the sedimentation and diffusion constants listed in Table II. The partial specific volume was assumed to be 0.75. The particle weights calculated from sedimentation and diffusion data and those from the viscosity and diffusion data show closer agreement in most instances when the particles are assumed to be disks rather than rods (Table III). The actual shapes are, of course, unknown. Following Polson[3] and Lauffer[25] the particle weights from viscosity and diffusion data were found from the equation

$$M = \frac{K(f_0/f)^3}{D^3V}$$

in which $K = (RT)^3/162\pi^2\eta^3N^2 = 2.41 \times 10^{-14}$ at 20°, or 3.67×10^{-14} at 25°. The axial ratios were found from the equations of Simha[15,16] and from these in turn the asymmetry factors were obtained from calculations after Perrin[26] and Herzog, Illig and Kudar[27] with the aid of graphs constructed from Table 4 on page 41 of reference 24.

When the final viscosities (Table II) and the roughly approximate particle weights from sedimentation and diffusion (Table III, Column 9) of the stabilized DnEa preparations are compared, the following relations seem apparent:

The particle weights of proteins produced by the same denaturant vary as their final viscosities.

For the acid-DnEa series, relatively small differences in viscosity accompany large differences in particle size. This is shown by a comparison of the final viscosities and particle weights of DnEa 16 (1.078 and 400,000, respectively) and DnEa 21 (1.117 and 800,000, respectively)(Tables II and III).

In contrast, in the alkali-DnEa series, only small differences in particle size were found for large differences in viscosity. The aggregated (on the basis of viscosity[1a]) DnEa 30, having a viscosity far greater than any of the other products studied in the ultracentrifuge, had a particle weight of only 500,000. It was thus hardly twice as large as DnEa 24 (particle weight 300,000) but had a much greater final viscosity. In conjunction with the low electrophoretic mobility, these differences in particle size in relation to viscosity indicate fundamental differences between alkali-DnEa and acid- or heat-DnEa, possibly interpretable according to the limited data of Tables II and III as a reflection of the smaller size of alkali-DnEa aggregates at equivalent dissymmetries.

Since in agreement with Rothen,[13] the sedimentation constant of heat-DnEa is also large, it would now appear that the tendency of several types of DnEa to form aggregates, even when their solutions have reached equilibrium, is well established. Whether or not the liberated —SH groups play a part in this new property is not known, but it is possible that a closer study of this property, not shown by native Ea, would reveal the nature of reactive groupings or centers liberated during denaturation, and so throw light on the as yet obscure mechanism of the process.

If it be assumed that denaturation involves no change in molecular weight[13,28] as distinguished from particle weight, a "normal" stabilized acid-DnEa is a polymer of at least eight DnEa molecules. It is probable, also, that "normal" alkali-DnEa is polymerized to nearly the same degree, as indicated in the preceding paragraph.

In preceding sections it was noted that a "normal" acid-DnEa exhibited a high initial viscosity which decreased rapidly during the first two to three weeks of aging and finally became constant after nine to twelve months. A very rough estimate of the particle weight of acid-DnEa 32,[1a] about 4×10^6, was obtained as early as possible during the aging process. The diffusion "constant" increased during the run, the values being 0.6, 1.1 and 1.75×10^{-7}, at the end of the second, third and fourth days (4th, 5th, and 6th days of aging). The first of these corresponded most closely with the time at which the sedimentation constant was determined and is listed in Table II. After aging one week the solution of DnEa 32 was no longer opalescent. The sedimentation and diffusion runs were re-

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(25) M. A. Lauffer, *Science*, **87**, 469 (1938); *J. Biol. Chem.*, **126**, 443 (1938).

(26) F. Perrin, *J. Phys. Rad.*, **7**, 1 (1936).

(27) R. O. Herzog, R. Illig and H. Kudar, *Z. physik. Chem.*, **167A**, 329 (1933).

(28) N. F. Burk and D. M. Greenberg, *J. Biol. Chem.*, **87**, 197 (1930).

peated after three weeks. At this time the diffusion constant showed no drift and the particle weight was calculated to be about 700,000, indicating that the product had split into units about one-sixth the size indicated after aging three to four days. At the end of two years, the particle weight was reduced to about 400,000.

From these observations on acid-DnEa and from the progressive decrease in opalescence, salt-sensitivity, and precipitability in the antigen-excess region with anti-Ea rabbit serum, it appears probable that the limited decrease in viscosity exhibited during aging by solutions of those lots of alkali- or heat-DnEa which were abnormally aggregated was also due to a splitting of the aggregates.

The results also point to the possibility that aggregation due to excess salt or prolonged standing in the isoelectric state involves linkages less easily dissociated than those formed in the characteristic partly reversible aggregation due to hydrochloric acid. When the former type of aggregation was imposed upon acid-, heat- or alkali-DnEa, aggregation was never reversed to the same extent as was the initial aggregation of optimally prepared acid-DnEa, as shown by the smaller regressions in viscosity on aging.

Summary

1. The fluidities of several lots of acid-, alkali- and heat-DnEa were determined during aging periods of several months and axial ratios were calculated.

2. Before aging, periods exceeding about sixteen hours in the isoelectric state lowered the fluidity of acid-DnEa but had no effect after about one month's aging. The fluidity of solutions of acid-DnEa increased on aging, with variation both in the initial fluidities and in the time required to constant fluidity. Salt in excess of 0.02 *M* or exposure to 37° decreased the fluidity and increased the opalescence of lots of acid-DnEa aged to constant fluidity.

3. The fluidity of "normal" alkali-DnEa was relatively high and changed little on aging. Long standing in the isoelectric state during isolation greatly decreased the fluidity and increased the opalescence and salt-sensitivity of undegraded alkali-DnEa, but these effects decreased with the extent of degradation.

4. The fluidity of Ea denatured by heat in the absence of salt remained constant on aging. Even low concentrations of salt during heat denaturation resulted in the formation of highly viscous products, the fluidities of which changed only slightly on aging.

5. The tendency of any type of DnEa to aggregate on exposure to excess salt or to 37° could be predicted roughly from the slope of its fluidity–concentration line.

6. Acid-, alkali- and heat-DnEa, purified by isoelectric precipitation and aged until their viscosities became constant, were found to be aggregates of from roughly 5 to 20 DnEa molecules. Those which were polymers of more than 5 to 12 were known to have been aggregated in excess of normal.

7. The decrease in viscosity of "normal" acid-DnEa on aging was shown to be due to disaggregation. Most of the splitting occurred during the first 2 to 3 weeks and corresponded with the time during which the viscosity decreased most rapidly and the solutions lost their opalescence.

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Denatured Egg Albumin. III. Quantitative Immunochemical Studies on Crystalline Egg Albumin Denatured in Various Ways[1]

BY CATHERINE F. C. MACPHERSON AND MICHAEL HEIDELBERGER

Qualitative serological studies[2] on crystalline egg albumin (Ea) denatured by heat, acid, shaking, or supersonic waves have failed to show significant differences in the behavior of the products with antiserum to denatured egg albumin (DnEa). While heat-, acid- or surface-DnEa were found to retain some of the original specificity, alkali-DnEa was reported to have lost its power to give cross reactions with antiserum to egg albumin and Ea did not cross-react with antiserum to alkali-DnEa.[2a] The alkali-DnEa used, however, had been denatured under conditions now known to cause degradation.[3]

In this paper quantitative immunochemical studies are presented on most of the products described in the first two papers of the series[3,4] and the differences found among them are discussed in terms of the type, degree of aggregation, and extent of degradation of the preparations.

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(3) C. F. C. MacPherson and M. Heidelberger, THIS JOURNAL, **67**, 574 (1945).

(4) C. F. C. MacPherson, M. Heidelberger and D. H. Moore, *ibid.*, **67**, 578 (1945).