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

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.

(1) The work reported in this series of communications was carried out in part under the Harkness Research Fund of Presbyterian Hospital and is to be 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.

(2) (a) H. Wu, C. Ten Broeck, and C. P. Li, *Chinese J. Physiol.*, **1**, 277 (1927); (b) E. W. Flosdorf and L. A. Chambers, *J. Immunol.*, **28**, 297 (1935).

(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).

TABLE I

ADDITION OF INCREASING AMOUNTS OF VARIOUS DnEa PREPARATIONS TO 1.0 ML. ANTI-ACID-DnEa SERUM 7673, 1:1, AT 37°

Preparation added	Antigen N added, mg.	Antigen N pptd., mg.	Total N pptd., mg.	Antibody N by diff., mg.	Ratio of antibody N to antigen N in ppt.	Tests on supernatants
Acid-DnEa 16	0.021	Total[a]	0.254	0.233	11.0	Excess A (antibody)
Acid-DnEa 16	.042	Total[a]	.425	.383	9.1	Excess A
Acid-DnEa 16	.083	Total[a]	.522	(.439)	(5.3)	No A, trace DnEa
Acid-DnEa 16	.168	0.14[b,c]	.55	.41	3.0	Excess DnEa
Acid-DnEa 16	.249	....	.49	...	...	Excess DnEa
Same after standing in 1.5% NaCl 2.5 mos.	.166	....	.60	...	...	Excess DnEa
Acid-DnEa 17	.048	Total[a]	.423	.375	7.8	Excess A
Acid-DnEa 17	.143	....	.57	...	...	Excess DnEa
Acid-DnEa 17	.222	0.15[b]	.52	.37	2.5	Excess DnEa
Acid-DnEa 21	.065	Total[a]	.500	.435	6.7	No A or DnEa
Acid-DnEa 21	.195	0.15[b,c]	.56	.41	2.7	Excess DnEa
Heat-DnEa 29	.048	Total[a]	.419	.371	7.7	Excess A
Heat-DnEa 29	.144	Total[b,c]	.578	.434	3.0	<0.001 mg. DnEa N
Heat-DnEa 29	.224	....	.63	...	...	Excess DnEa
Heat-DnEa 25	.043	Total[a]	.343	.300	7.0	Excess A
Heat-DnEa 25	.172	Total[b,c]	.604	.432	2.5	<0.001 mg. DnEa N
Alkali-DnEa 30	.050	Total[a]	.479	.429	8.6	No A or DnEa
Alkali-DnEa 30	.100	0.09[b,c]	.52	.43	4.8	Excess DnEa
Alkali-DnEa 30	.233	0.04[b,c]	.41	.38	9.5	Excess DnEa
Alkali-DnEa 24	.051	Total[a]	.470	.419	8.2	No A or DnEa
Alkali-DnEa 24	.067	....	.505[c]	...	...	Excess DnEa
Alkali-DnEa 24	.152	0.07[b]	.47	.40	5.7	Excess DnEa
Alkali-DnEa 24	.202	....	.38	...	...	Excess DnEa
Alkali-DnEa 18	.048	Total[a]	.467	(.419)	(8.7)	No A, trace DnEa
Alkali-DnEa 18	.144	0.04[b]	.38	.34	8.5	Excess DnEa
H_2O-DnEa	.049	Total[a]	.484	.435	8.9	No A or DnEa
H_2O-DnEa	.229	0.03[b,c]	.34	.31	10.3	Excess DnEa
Comparative Data for the Egg Albumin-Anti-Egg Albumin System[d]						
Ea	.0089	Total	.201	.192	21.5	Excess A
Ea	.0296	Total	.443	.414	14.0	No A or Ea
Ea	.042	Total	.481	.439	10.4	No A or Ea
Ea	.059	0.058	.498	.440	7.5	0.001 Ea N
Ea	.071	.061	.466	.405	6.6	Excess Ea
Ea	.089	....	.310	...	...	...

[a] Assumed, in accordance with the behavior of other protein-antiprotein systems. [b] From analysis of supernatants for DnEa as described in Ref. 8. [c] Figure obtained from a single analysis. [d] Values for the Ea–anti-Ea system were obtained from Table II, ref. 8, and were recalculated to a maximum total nitrogen content of 0.5 mg. per ml.

Figures in the third decimal place are uncertain, but are included, except for the less accurate region of antigen excess, as they represent the actual experimental data used in the calculation of ratios. In most instances the analyses were carried out on larger volumes of serum and were calculated to 1.0 ml. for insertion in the table.

Experimental

1. **Immune Sera.**—Antisera were prepared against acid-DnEa 16, acid-DnEa 17, and alkali-DnEa 23 and 28. Before each lot of antigen was made up, the aged DnEa was isoelectrically precipitated as many times as necessary to yield a supernatant negative with trichloroacetic acid in order to ensure the absence of Ea.[3] Rabbits were injected intravenously with neutral suspensions of alum-precipitated DnEa containing 3 mg. of protein per ml. The initial doses of 1 to 1.5 mg. were increased to 5 to 8 mg. during the first two weeks, the average course consisting of four injections a week for four weeks. Antisera were generally weak, averaging 0.5 mg. of antibody N per ml.

2. **Analytical Methods.**—The quantitative analyses[5,6,7,8] in anti-DnEa sera were carried out after the viscosities of most of the lots of DnEa used as antigen had ceased to change appreciably. The protein solutions were diluted to approximately 0.1 mg. N per ml. with 0.5% saline containing 1:10,000 Merthiolate.[9] DnEa prepara-

(5) M. Heidelberger and F. E. Kendall, *J. Exptl. Med.*, **55**, 555 (1932).

(6) M. Heidelberger, F. E. Kendall and C. M. Soo Hoo, *ibid.*, **58**, 137 (1933).

(7) M. Heidelberger and F. E. Kendall, *ibid.*, **61**, 559 (1935).

(8) M. Heidelberger and F. E. Kendall, *ibid.*, **62**, 697 (1935).

(9) Manufactured by Eli Lilly & Co., Indianapolis, Ind.

TABLE II

ADDITION OF INCREASING AMOUNTS OF VARIOUS DnEa PREPARATIONS TO 1.0 ML. OF POOLED ANTI-ALKALI-DnEa SERA 7743 AND 7753 AT 37°

DnEa added	DnEa N added, mg.	DnEa N pptd., mg.	Total N pptd., mg.	Antibody N by diff., mg.	Ratio of antibody N:DnEa N in ppt.	Tests on supernatants
Alkali-DnEa 23	0.011	Total[a]	0.168	0.157	14.6	Excess A
Alkali-DnEa 23	.024	Total[a]	.304	.280	11.7	Excess A
Alkali-DnEa 23	.068	Total[b,c]	.459	.391	(6)	<0.001 mg. DnEa N
Alkali-DnEa 23	.150	....	.45	...	..	Excess DnEa
Alkali-DnEa 23	.180	....	.42	...	..	Excess DnEa
Alkali-DnEa 30	.049	Total[a]	.430	.381	7.8	No A or DnEa
Alkali-DnEa 30	.163	0.14[b]	.47	.33	2.4	Excess DnEa
Alkali-DnEa 24	.048	Total[a]	.419	.371	7.8	No A or DnEa
Alkali-DnEa 24	.159	0.13[b,c]	.43	.30	2.3	Excess DnEa
Alkali-DnEa 18	.048	Total[a]	.402	.354	7.4	Excess A, trace DnEa
Alkali-DnEa 18	.160	0.12[b,c]	.34	.22	1.8	Excess DnEa
H_2O-DnEa	.049	Total[a]	.427	.378	7.7	Excess A
H_2O-DnEa	.163	0.14[b]	.45	.31	2.2	Excess DnEa
Acid-DnEa 16	.042	Total[a]	.361	.319	7.7	Excess A
Acid-DnEa 16	.166	....	.53	...	..	Excess DnEa
Acid-DnEa 16	.249	....	.45	...	..	Excess DnEa
Acid-DnEa 17	.049	Total[a]	.378	.329	6.7	Excess A
Acid-DnEa 17	.163	0.15[b,c]	.48	.33	2.2	Excess DnEa
Heat-DnEa 29	.042	Total[a]	.333	.291	7.0	Excess A
Heat-DnEa 29	.166	0.16[b]	.52	.36	2.3	Excess DnEa
Heat-DnEa 29	.249	....	.51	...	..	Excess DnEa

[a] Assumed. [b] Supernatants analyzed for DnEa according to ref. 8. [c] Figure obtained from a single analysis.

tions which became opalescent on dilution with saline (lots 21, 25, 29 and 30) were diluted immediately before addition to the sera. The reaction times and temperatures used were varied widely in preliminary studies because of the tendency of DnEa to aggregate in the presence of physiological concentrations of electrolyte.[4] In the region of antibody excess the reaction between DnEa and anti-DnEa was complete in 48 hours at 0–5°. However, when mixtures containing excess antigen were analyzed either at 37 or 0°, more nitrogen was precipitated the longer the mixtures were allowed to stand. Since the same amounts of total nitrogen were precipitated in this region whether the mixtures were allowed to stand at 37° for three hours, at room temperature for two days, or in the ice box at 0–5° for one week, conditions usually more than adequate for complete precipitation of homologous antigen-antibody combinations, the shorter reaction time at 37° was adopted as a matter of convenience. Appropriate amounts of antigen and serum were mixed and allowed to stand at 37° for three hours; the precipitates were then centrifuged off at room temperature and washed twice at 0° with 0.5% saline containing 1 ml. of 1% saponin per 100 ml. This effectively abolished the film formation which occurred during the washing of precipitates formed in the region of antigen excess.[3,10]

In Table I, results are given for the calibration of an anti-acid-DnEa serum with its homologous antigen (DnEa 16) up to the region of slight inhibition. Data on the interaction of several other lots of DnEa with this serum are included and, in addition, an analysis with a dilute solution of DnEa 16 in 1.5% saline which had been allowed to stand for two and one-half months in the ice box. In Table II, the results of a similar study of the reactions of the various types of DnEa in anti-alkali-DnEa serum are listed. The results in Tables I and II are plotted in Fig. 1 on a comparative basis after calculation to 0.5 mg. total nitrogen precipitated, while the experimental data in the tables are plotted in Fig. 2. Data on the cross-reactions in anti-Ea serum are plotted in Fig. 3.

Studies on the extent of the cross-reaction of the various lots of DnEa with anti-Ea serum were carried out at 37° for twenty-four hours, since supernatants returned to the incubator after the specific precipitates were centrifuged off showed no further change after another seventy-two hours. The supernatants and washings of the DnEa–anti-Ea precipitates were sometimes opalescent, rendering interpretation of such analytical results somewhat uncertain. The degree of opalescence was directly related to the concentration of DnEa in the reaction mixtures and was most marked in the case of alkali-DnEa. For these comparisons the freshly diluted antigen solutions usually contained 0.5 mg. N per ml. for alkali-DnEa and 0.2 mg. N per ml. for acid- and heat-DnEa. Antigen blanks were usually negative except those of alkali-DnEa 30 (0.02 mg. N) and acid-DnEa 33 (0.04 mg. N) before the preparations had aged. In addition to the Merthiolate in both antigens and sera, enough 5% phenol was added to each measured aliquot of serum to make the final concentration about 0.2% in order to ensure sterility.

Quantitative results are reported only on sera on which extensive comparative studies were made. The various lots of DnEa were compared in anti-DnEa sera at points in the region of antibody excess near the equivalence zone and at one or two points in the region of antigen excess. Several additional sera were studied and the discussion of the general characteristics of the DnEa–anti-DnEa system is based on results obtained with these sera as well.

The amounts of nitrogen precipitated from anti-Ea sera by three lots of alkali-DnEa aggregated in excess of "normal" (nos. 24, 28 and 30) and "normal" acid-DnEa 33 were determined before appreciable aging had occurred and after aging had progressed for one to three months. The other lots of DnEa were compared in anti-Ea sera when their viscosities were substantially constant.

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

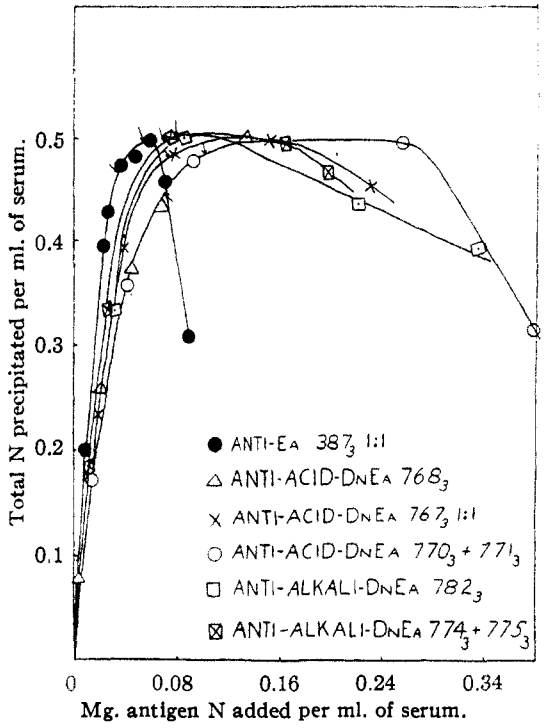

Fig. 1.—Comparison of precipitin reaction of Ea–anti-Ea system with acid-DnEa–anti-acid-DnEa and alkali-DnEa–anti-alkali DnEa. All data recalculated to 0.5 mg. maximum total N. Arrows on each curve mark off equivalence zone.

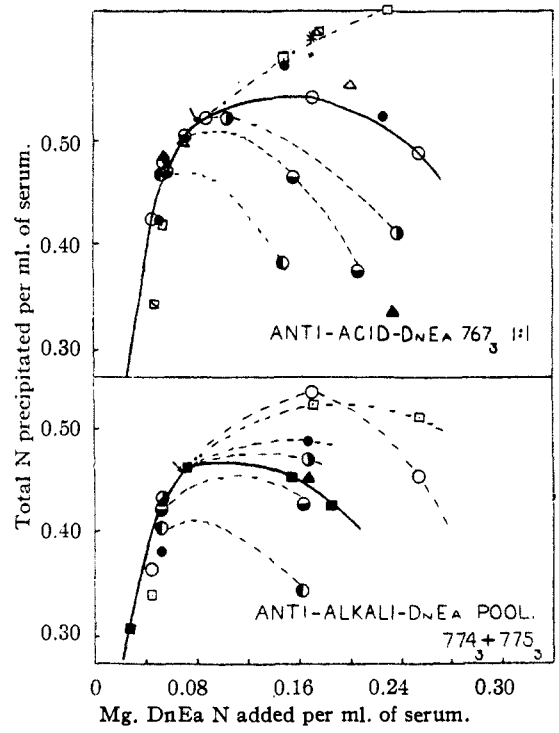

Fig. 2.—Key to chart: ⊙, acid-DnEa 16; ⁎, acid-DnEa 16 (1.5% NaCl); ●, acid-DnEa 17; △, acid-DnEa 21; ⊡, heat-DnEa 29; ▨, heat-DnEa 25; ▲, H_2O-DnEa; ◑, alkali-DnEa 30; ◒, alkali-DnEa 24; ◐, alkali-DnEa 18; ■, alkali DnEa 23. Total N precipitated from 1.0 ml. of anti-DnEa rabbit sera by increasing amounts of various preparations of DnEa. Solid lines represent N precipitated by the antigens used for immunization; i.e., acid-DnEa 16 in the case of anti-acid DnEa 7673 and alkali-DnEa 23 in the case of the anti-alkali-DnEa pool. Arrows show the points of approximate equivalence of homologous reaction. Ordinates do not start from origin of curves at 0,0.

Results and Discussion

Comparison of Different Kinds of DnEa in Anti-Acid-DnEa Sera

The various lots of DnEa were found to be equivalent when they were compared against anti-acid-DnEa serum in the region of antibody excess near the equivalence zone. The type or the degree of aggregation or degradation appeared to have no influence on the results (Fig. 2), in contrast to the evident effect of these factors in the region of antigen excess, in which small quantitative differences were found among the various products. These findings may be summarized as follows:

Acid-DnEa.—The reaction between acid-DnEa and its homologous antiserum differed from that between Ea and anti-Ea[8] in several respects (Table I and Fig. 1). Throughout the reaction range the ratios of antibody N: DnEa N in the specific precipitates were considerably lower, the region between the point at which antigen first appeared in excess and that at which maximum precipitation of nitrogen occurred was broader, and inhibition of precipitation by excess antigen required greater quantities of DnEa. While a quantity of acid-DnEa such as to leave it in slight excess in the supernatant precipitated most of the antibody, from two to three times this amount of DnEa was generally required to reach maximum precipitation (total N).

Aggregated acid-DnEa (DnEa 21 and a portion of DnEa 16 aggregated by standing in 1.5% saline for two and a half months) precipitated more total N in the region of antigen excess than did the homologous antigen, "normal" DnEa 16. The larger amounts of nitrogen precipitated by the aggregated antigens appeared due to the larger masses of aggregate attached to the DnEa molecules in direct chemical combination with the antibody. Tests on supernatants in the region of antigen excess actually showed that when "normal" and aggregated lots of acid-DnEa were compared at equal antigen nitrogen concentrations, less antigen remained in the supernatants of the latter.

Alkali-DnEa.—Preparations of alkali-DnEa precipitated less nitrogen from anti-acid-DnEa serum in the region of antigen excess than did the homologous antigen. Accordingly, the ratios of antibody N: DnEa N in the specific precipitates formed in the region of antigen excess were of the order of 6–10 for alkali-DnEa in con-

trast to the much lower values (2–3) for acid- and heat-DnEa (Table I). For this reason, although the total nitrogen precipitated by acid-DnEa was greater, the antibody nitrogen precipitated by acid- and alkali-DnEa was practically the same. These differences in ratios permitted a serological distinction to be made between alkali-DnEa on the one hand, and acid- and heat-DnEa on the other. The smaller amounts of nitrogen precipitated by alkali-DnEa 24 and DnEa 18, however, appeared to be a result of the degradation to which they were subjected.[3]

Heat-DnEa.—Both lots of heat-DnEa used were aggregated in excess of normal, DnEa 29 having a particle weight of about 900,000[4] and DnEa 25 being very much larger. Yet both precipitated similar amounts of N in the region of antigen excess and more than the aggregated acid-DnEa 21 which was of approximately the same size as DnEa 29.

H_2O-DnEa[3].—This product, which had separated on long standing from an isoelectric solution of Ea, resembled alkali-DnEa in the amount of nitrogen it precipitated from anti-acid-DnEa sera in the region of antigen excess (Table I and Fig. 2).

Comparisons in Anti-Alkali-DnEa Sera

Two anti-alkali-DnEa sera were pooled after the rabbits had been given three courses of injections. The sera contained less antibody than the anti-acid-DnEa sera.

Just as in anti-acid-DnEa serum, all varieties of DnEa were found to be equivalent in anti-alkali-DnEa serum in the region of antibody excess near the equivalence zone. The ratios of antibody N:DnEa N in the precipitates in the region of antigen excess were low and of the same order for all antigens (Table II), but minor differences were again noted (Table II and Fig. 2).

Alkali-DnEa.—The reaction between alkali-DnEa and its homologous antiserum resembled the acid-DnEa-anti-acid-DnEa system in having similar ratios of antibody N:DnEa N in the specific precipitates and a broad antigen excess region over which inhibition of specific precipitation by excess DnEa occurred gradually. It differed from the acid–DnEa system in that the region between the points at which antigen first appeared in excess and maximum precipitation of N was narrow.

The aggregated but undegraded alkali-DnEa 30 precipitated the same amount of nitrogen as did the homologous antigen, DnEa 23. The degraded lots of alkali-DnEa (24 and 18) precipitated less nitrogen in proportion to their degree of degradation, just as in anti-acid-DnEa serum.

Acid- and Heat-DnEa.—Acid-DnEa 16 and heat-DnEa 29 precipitated the most total nitrogen in the region of antigen excess but inhibition of precipitation began more quickly with the acid-DnEa as quantities of antigen were increased.

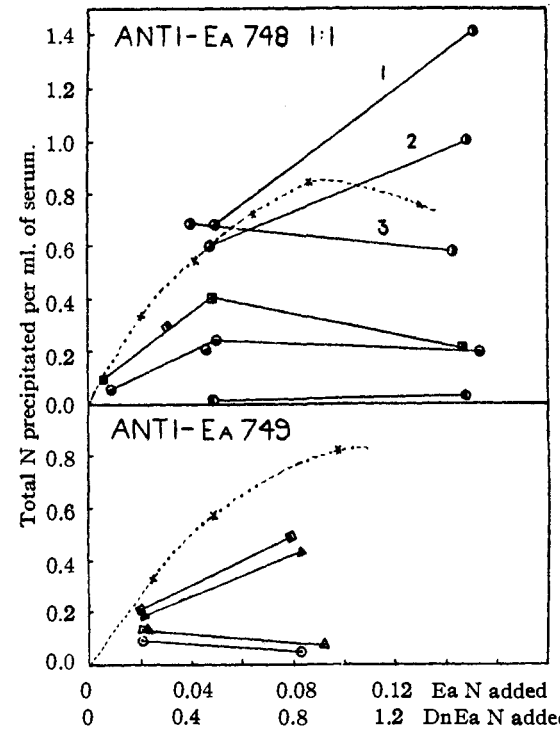

Fig. 3.—Solid lines without numbers represent total N precipitated from 1.0 ml. of anti-Ea rabbit sera by various preparations of DnEa after their viscosities had become substantially constant. Lines 1, 2 and 3 show decreasing total N precipitated by abnormally aggregated alkali-DnEa 30 before aging and after aging for one and three months. The homologous Ea-anti-Ea reaction is shown by broken curves. Note that amounts of Ea and DnEa shown differ ten-fold. Key to chart: ×, Ea; ◑, alkali-DnEa 30; ◨, alkali-DnEa 28; ◒, alkali-DnEa 24; ◐, alkali-DnEa 18; ▲, H_2O-DnEa; ◧, TCA-DnEa; □, heat-DnEa 29; ⊙, acid-DnEa 16; △, acid-DnEa 21.

H_2O-DnEa.—This product resembled alkali-DnEa.

Comparisons in Anti-Ea Sera

Cross-reactions of the various lots of DnEa with anti-Ea sera are plotted in Fig. 3. Quantities of DnEa roughly ten times those of the homologous Ea were required to produce comparable, and in some instances, appreciable precipitation. This may be considered a reflection of the change in immunological specificity caused by the primary process of denaturation itself. The changes due to aggregation are relatively minor (lines 1, 2, 3), and in the opposite direction.

Acid- and Heat-DnEa.—It will be noted that the aged preparations of acid- and heat-DnEa cross-reacted to about the same slight extent. The differences found in the nitrogen precipitated by acid-DnEa 33 before and after it had aged for two months (Table III) were presumably due to the large differences in the size of the aggregates

at these times, as judged by the behavior of similar preparations.[4]

TABLE III

AMOUNTS OF NITROGEN IN MG. PRECIPITATED FROM 1.0 ML. OF ANTI-Ea POOL K (0.80 MG. ANTIBODY N PER ML.) AT 37° BY "NORMAL" ACID-DnEa 33 BEFORE AND AFTER AGING

DnEa N added	Total N precipitated by DnEa 33	
	Before aging	Aged for two months
0.106	0.198	0.092
.532	.750	.210

Alkali-DnEa.—The cross-reaction of alkali-DnEa was influenced by the extent of aggregation and degradation, just as in its reaction with anti-DnEa sera. The undegraded but aggregated alkali-DnEa 30 (particle weight about 500,000) precipitated more N than any other antigen and the amounts of nitrogen precipitated decreased markedly as the preparation aged. DnEa 28 and DnEa 24, less aggregated than DnEa 30, also precipitated less nitrogen on aging but the change was not nearly as great as in the case of DnEa 30. The severely degraded alkali-DnEa 18, prepared under conditions similar to those used in ref. 2a, showed practically no reaction with anti-Ea serum, as found by Wu, Ten Broeck, and Li.[2a]

If diminution in opalescence and decrease in the viscosity and in the amount of nitrogen precipitated from anti-Ea sera may be interpreted to indicate a decrease in size of undegraded alkali-DnEa as they did in the case of acid-DnEa,[4] behavior in anti-Ea sera would provide a more sensitive means than viscosity for following changes in size of alkali-DnEa particles with age, since the viscosity of their solutions decreased only slightly on aging.[4]

H_2O- and TCA-DnEa[3].—These products (among the least salt-sensitive of the various lots of DnEa studied) gave cross-reactions in anti-Ea serum similar to that given by the undegraded but slightly aggregated alkali-DnEa 28.

Data on the cross-reaction of Ea in antiserum to acid-DnEa were given in a preliminary report[11] and differences from both homologous reactions were shown. Again, large differences were noted in the quantities of Ea and DnEa necessary to precipitate the antiserum, in this instance far more Ea being required. Qualitative tests showed Ea to react with anti-alkali-DnEa serum, as well, contrary to Wu, Ten Broeck, and Li.[2a]

On the whole, the various types of DnEa showed a remarkably uniform serological behavior, and in the region of antibody excess, in which all antigen added was precipitated, the various products appeared identical. However, in the region of antigen excess, as noted above, differences were found. These appeared ascribable largely to variations in the extent of aggregation and degradation of the preparations, especially within any one type. Only in the case of alkali-DnEa, with its relatively high antibody-antigen combining ratios in the region of antigen excess, did a limited serological differentiation between this product and others such as acid- or heat-DnEa appear possible. This should be checked by further work, however, for it is precisely in the region of antigen excess that analytical errors and uncertainties are greatest.

Recently, Rothen and Landsteiner,[12] using a precipitation technique, found that the species specificity of denatured albumins was lower than that of the native proteins, but certainly not abolished, as has been stated.[13] It was also reported[12] that films made from native Ea and heat-DnEa reacted similarly with antisera to the native and heat-denatured proteins. In contrast, DnEa in solution was found to react differently with these sera. These contradictory findings were probably due to the lowered specificity of serological reactions in films indicated by the data in reference 12.

The relation of the present work to that of Erickson and Neurath[14,15] is not clear. The criterion for denatured protein used by the present writers was insolubility at the isoelectric point, native Ea being soluble under the same conditions. This is the classical definition, but may require modification. However, in the light of other recent investigations, mentioned below, it would appear that more exact information on native as well as denatured proteins is required in order to decide to what extent the properties of different preparations may vary and still permit of classification of particular samples of the protein as native. For example, the main component of Ea has been found to change over into an electrophoretic satellite[16] with age.[17] Different samples of Ea were denatured at different rates by acid[3] and samples of tobacco mosaic virus behaved similarly on denaturation by urea.[18] Astbury, Dickinson and Bailey[19] noted that under certain conditions the crystals of the seed globulin, excelsin, gave an X-ray pattern with some of the features of the X-ray pattern of β-keratin. Lundgren and Williams[20] have presented evidence of what they believed to be un-

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folded forms of hog thyroglobulin, thymus nucleohistone and diphtheria antitoxin under particular conditions, in the ultracentrifuge along with the native folded forms. These so-called X-proteins seemed to be intermediate between the native and denatured forms.[20a]

Steinhardt's work on the heat-denaturation of pepsin[21] and the re-examination of the activation energies of denaturation by Eyring and Stearn[22] have modified the conception of the denatured state as one of such great configurative variety as suggested by the earlier calculations of Mirsky and Pauling.[23] The definite differences noted between alkali-DnEa and the other forms, as well as the antigenicity and characteristic specificity of DnEa, are incompatible with the characterization of the denatured state as a "debris of peptide chains."[19] Even the size of the aggregates of DnEa in aqueous solution is now shown to be controllable, so that the manner of aggregation, also, would appear to be an orderly process.

(20a) *Cf.* also H. Neurath, J. P. Greenstein, F. W. Putnam and J. O. Erickson, *Chem. Rev.*, **34**, 157 (1944).

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Summary

1. Serological studies of DnEa prepared in a variety of ways showed that all preparations behaved alike in that part of the reaction range characterized by antibody excess.

2. Alkali-DnEa, in the reaction region of antigen excess, showed higher antibody-antigen ratios than did acid- or heat-DnEa. Within any one type the results were modified by aggregation and degradation. Decrease in the size of DnEa aggregates on aging lessened the amount of nitrogen precipitated from anti-Ea serum.

3. The quantitative immunochemical technique has served to supplement information gained by parallel chemical and physical studies of DnEa. Definite structural entities are indicated for DnEa, rather than a disordered state.

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Substituted Diphenylarsinic Acids and their Reduction Products

BY HUGO BAUER

Organic derivatives of arsenic hitherto investigated for their action against streptococci proved to be inactive. The strong bactericidal activity of bis-(4-aminophenyl)-sulfone[1] and of 4-nitro-4'-aminodiphenylsulfone[2] suggested the preparation of arsenicals of analogous structure. Diphenylarsinic acids, substituted in para position by amino and nitro groups, and their reduction products, were synthesized and tested for antistreptococcal action.[3] For starting material, 4-nitro-4'-aminodiphenylarsinic acid was prepared by action of 4-nitrodiazobenzene upon 4-acetylaminophenylarsine oxide, using the Sakellarios modification of the Bart method.[4] Reduction of the nitro group served as a convenient method for preparing bis-(4-aminophenyl) arsinic acid which hitherto was obtained in small yield only as a by-product in preparing arsanilic acid.[5] A series of reduction products was prepared employing the usual procedures. Secondary arsyl oxides and hydroxides were obtained by reduction of the corresponding arsinic acid with sulfur dioxide in presence of iodine; they were amorphous, but one of them (V) could be obtained in crystalline form containing benzene of crystallization. For the preparation of secondary diarsyls, hypophosphorous acid in presence of potassium iodide was employed. They were obtained as crystalline powders.

The bis-(4-aminophenyl)-arsyl hydroxide (X) and the corresponding arsyl chloride (IX) decompose readily in acid solution with formation of the tertiary tris-(4-aminophenyl)-arsine (XIII). This reaction is analogous to the formation of XIII from 4-aminophenyl-arsine oxide, described by Ehrlich and Bertheim.[6] From the arsine XIII, the corresponding arsine oxide (XIV) could be obtained by oxidation with iodine.

The description of 4,4',4'',4'''-tetraaminotetraphenylarsyl oxide given in the earlier publication[3] should be disregarded because we were then dealing with a decomposition product.

The compounds tested by S. M. Rosenthal[3] against hemolytic streptococci in mice, are shown in the table. 4-Nitro-4'-aminodiphenylarsinic acid showed some activity which was increased by acetylation. The activity of the acetyl derivative (I) was approximately the same as that of sulfanilamide, but curative effects were obtained only when the drug was administered in amounts close to the tolerated dose. The corresponding arsyl oxide and hydroxide (V and VI)

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