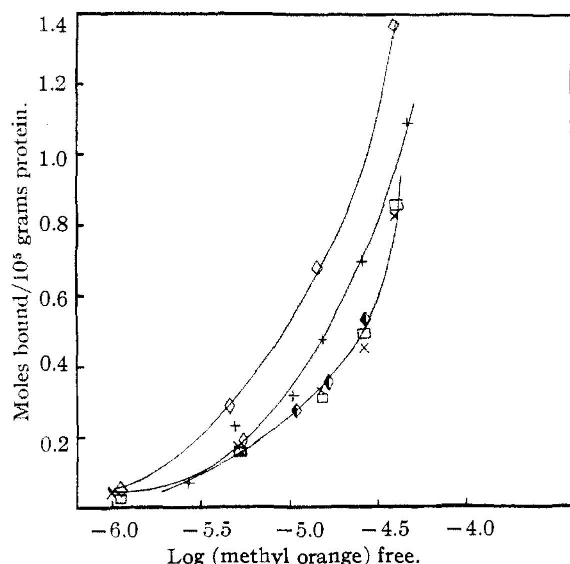


Fig. 4.—Methyl orange bound by DnEa and deaminated Ea fractions—key to chart: ◇ DnEa 105, ◐ DnEa 105 Deam-SH, □ 5A, × 5A Dn, + 8B Dn.

in Table IV. Here, too, a definite difference is shown between acid DnEa and heat DnEa on the one hand, and an A fraction on the other. In 1.5 *N* NaCl solution the A fraction remains soluble whereas the DnEa preparations precipitate to the extent of 60%. This would indicate that the A fraction does not exist in a form as fully extended or as subject to aggregation as the other DnEa preparations, in accord with the nitroprusside tests and the particle weights. In its salt sensitivity, also, fraction 5ADn appeared to be more unfolded or aggregated than 5A. Whereas 5A was opalescent in 2.0 *M* NaCl, 5ADn precipitated to the extent of 24%. Acid denatured 8B showed less tendency to aggregate than DnEa, in agreement with the viscosity data.

TABLE IV

EFFECT OF SODIUM CHLORIDE SOLUTIONS ON VARIOUS DENATURED PREPARATIONS. PER CENT. PROTEIN PRECIPITATED, 1 HR., 37°

| | 0.25 *M* | 0.5 *M* | 1.0 *M* | 1.5 *M* | 2.0 *M* | 2.5 *M* |
|---|---|---|---|---|---|---|
| 5A | *a* | *a* | *a* | *a* | *b* | 63 |
| 5A Dn | *a* | *a* | *a* | *b* | 24[c] | 76 |
| Acid DnEa 105 | *a* | *b* | *b* | 57 | 72 | 99 |
| 8B Dn | *a* | *a* | *b* | 31[c] | 69 | 88 |
| Heat Dn 27 | *b* | *b* | 63 | 88 | 94 | 98 |

[a] Clear solution. [b] Opalescent solution. [c] Supernatant opalescent after centrifugation.

9. **Binding of Methyl Orange.**—The extent of binding of the anion of methyl orange by approximately 0.2% protein solutions was measured at *p*H 6.8 by differential dialysis.[36] The samples were dialyzed against dye solutions ranging in concentration from $0.165 \times 10^{-5}$ to $8.40 \times 10^{-5}$ *M*. The results are summarized in Fig. 4. Neither Ea nor 8B bound dye, but DnEa did, in agreement with results and statements of others.[37]

When acid DnEa is deaminated, it has a reduced binding ability similar to that of the A and ADn preparations. 8BDn, which can be considered in a sense "isomeric" with deaminated DnEa, binds less than DnEa and more than its deaminated form. It is difficult to decide what part in these relationships is played by the decrease in cationic nitrogen and what part is due to differences in molecular configuration.

(36) I. M. Klotz, F. M. Walker and R. B. Piven, THIS JOURNAL, **68**, 1486 (1946).

(37) I. M. Klotz and J. M. Urquhart, *ibid.*, **71**, 1597 (1949).

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

# The Deamination of Crystalline Egg Albumin.[1,2] III. Quantitative Immunochemical Studies on Crystalline Egg Albumin and its Denatured and Deaminated Derivatives

BY PAUL H. MAURER AND MICHAEL HEIDELBERGER

The serological specificity of Ea is not influenced by the removal of about one-third of the free $-NH_2$ groups when the protein remains undenatured. In the denatured form, which shows a specificity different from that of Ea, removal of up to *ca.* 60% of $-NH_2$ from DnEa or A resulted in a scarcely significant decrease in the nitrogen precipitated from their homologous antisera. Changes in immunolgical specificity of Ea, DnEa, and fractions A and B due to physical aggregation or change of -S-S- to -SH linkages are relatively minor. The quantitative immunochemical data supplement information gained by parallel chemical and physical studies of Ea and its deaminated and denatured derivatives and lead to the conclusion that the products described have definite form and structure.

In previous papers[3,4] it was shown that the fraction B of partially deaminated egg albumin (Ea) soluble at the isoelectric point (i.e.p.), had many properties resembling those of native Ea. On the other hand, Fraction A, which was rendered insoluble at the i.e.p. during deamination and acquired many of the properties of denatured Ea (DnEa), nevertheless showed definite differences from Ea denatured, for example, by acid.

These resemblances and differences are further illustrated in the accompanying quantitative immunochemical studies. These show both the wide applicability and the limitations of this technique.

(1) Submitted by Paul H. Maurer in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Faculty of Pure Science, Columbia University.

(2) Presented before the 34th Annual Meeting of the American Association of Immunologists, Atlantic City, N. J., April 17–21, 1950.

(3) P. H. Maurer and M. Heidelberger, THIS JOURNAL, **73**, 2070 (1951).

(4) P. H. Maurer and M. Heidelberger, *ibid.* **73**, 2072 (1951).

## Experimental

1. **Immune Sera.**—Antisera were prepared against Ea,[5] acid DnEa,[6] and fractions A and B of deaminated lot 4, by injection of rabbits intravenously with neutral suspensions of alum-precipitated protein containing 1.5 mg. of antigen per ml. Initial doses of 0.5–1.5 mg. were increased to 4 mg. during the first 2 weeks and to 8 mg. by the end of the course which consisted of 4 injections a week for 4 weeks. Bleedings were taken about a week after the last injection.

Antisera to Ea and 4B contained about 2.5 and 1.5 mg. antibody N per ml., respectively. On the other hand, antisera to DnEa and 4A were generally weak, containing about 0.12 and 0.25 mg. antibody N per ml., respectively, in accord with observations that denatured proteins are less antigenic than the native forms from which they are derived.[6,7]

2. **Analytical Methods.**—Quantitative analyses in anti-4B sera were carried out as in the Ea–anti Ea system[5] and were allowed to stand 48 hours at 0°. A few analyses were done at 37° for comparison. Analyses in the inhibition zone in anti-Ea and anti-4B sera were run at 37° for 1 hour and then placed in the ice-box for 4 days with stirring each day.[5] Analyses in anti-4A sera were run as with anti-DnEa sera,[6] but no difference could be observed in total N precipitated whether Fraction A was diluted with 0.5% NaCl solution, as was always done in the case of DnEa[6] or with 0.9% NaCl, as is usual. Accurately measured solutions of antigen and serum were mixed and allowed to stand at 37° for 3 hr. The precipitates were centrifuged off at room temperature and washed twice at 0° with 0.9% NaCl solution. All washings were spun in a refrigerated centrifuge. In the case of the specific precipitates with DnEa as antigen 0.5% NaCl solutions containing 1 ml. of 1% saponin per 100 ml. were used as wash fluid. N in the precipitates was determined by the Markham modification of the micro-Kjeldahl procedure.[8] Cross reactions were generally run at the two concentrations indicated in the tables and figures.

## Results and Discussion

**1. Comparisons in Anti-Ea and Anti-4B Sera.**—Homologous and cross reactions of Ea and fraction B, whether in the oxidized (–S–S–) or reduced (–SH) forms, with calibrated anti-Ea (Fig. 1) and calibrated anti-B sera (Fig. 2) were indistinguishable up to the region of moderate antigen excess. These data furnish a rare example of the limitations of quantitative immunochemical techniques, for Ea and Fraction B were obviously different in their electrophoretic mobilities,[4] $-NH_2$ content, and other properties. However, when determinations of specifically precipitable N were made with large quantities of antigen far out in the inhibition zone, the B fraction always precipitated more N, that is, inhibited less. According to Cohn, Wetter and Deutsch[9] this might indicate a slight impurity in the B fraction. Along these lines, it is possible that a trace of Fraction A, conalbumin or other constituent of egg white not detectable by other methods had become more concentrated in the B fraction than in the original Ea used.

The data obtained indicate that in the native Ea–anti-Ea and B fraction–anti-B systems sero-

(5) M. Heidelberger and F. E. Kendall, *J. Exptl. Med.*, **62**, 697 (1935).

(6) C. F. C. MacPherson and M. Heidelberger, THIS JOURNAL, **67**, 585 (1945).

(7) H. Wu, C. TenBroeck and C. P. Li, *Chinese J. Physiol.*, **1**, 277 (1927).

(8) R. Markham, *Biochem. J.*, **36**, 790 (1942); E. A. Kabat and M. M. Mayer, "Experimental Immunochemistry," C. C. Thomas, Springfield, Ill., 1948.

(9) M. Cohn, L. R. Wetter and H. F. Deutsch, *J. Immunol.*, **61**, 283 (1949).

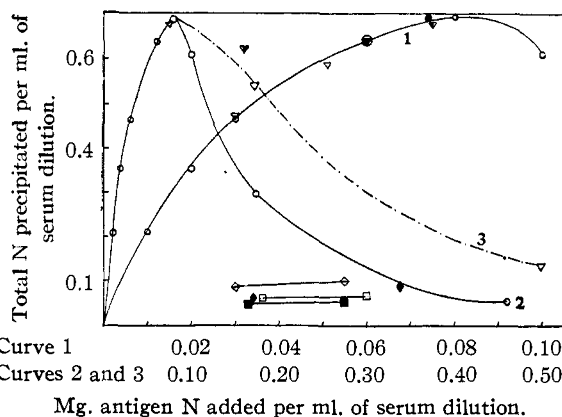


Fig. 1.—Homologous and cross precipitation with anti-Ea serum FK2(1 → 4). Key to chart: ○, Ea; ●, Ea at 37°; ▽, 4B; ▼, 4B at 37°; ▼, 5B; ▼, 8B SS; □, 5A; ■, 6A; ◇, DnEa 101; ◆, HtDn 27.

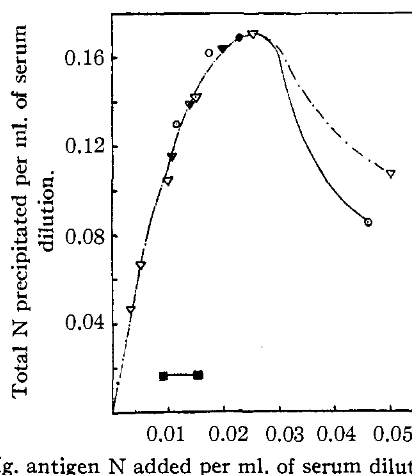


Fig. 2.—Homologous and cross precipitation with anti-4B serum $979_1$ (1 → 10). Key to chart: ▽, 4B; ▼, 4B at 37°; ▼, 3B; ▼, 5B; ○, Ea; ●, Ea at 37°; ■, 4A.

logical specificity is unaffected by the presence or absence of roughly one-third of the amino groups or by the state of the –S– groups as –S–S– or –SH. This confirms the work of Blumenthal[10] who found no difference between the –SH or –S–S– forms of Ea with anti-Ea sera. The urease–anti-urease[11] and ferritin–anti-ferritin[12] systems behave similarly.

Both acid DnEa and fraction A gave cross reactions with anti-4B and anti-Ea sera to the same slight degree, indicating that they are both denatured forms of Ea. This is also borne out by the cross reactions of A fractions with anti-acid DnEa sera discussed below.

**2. Comparisons in Anti-DnEa and Anti-A Sera.**—From the cross reactions of the A fractions in anti-acid DnEa sera (Fig. 3) and acid DnEa in anti-4A sera (Fig. 4) it is apparent that the two derivatives of Ea are different serologically although they are both denatured. It was shown previously that DnEa prepared by

(10) D. Blumenthal, *J. Biol. Chem.*, **113**, 433 (1936).

(11) L. Pillemer, E. E. Ecker, V. C. Myers and E. Muntwyler, *ibid.*, **123**, 365 (1938).

(12) A. Mazur, personal communication.

TABLE I

ADDITION OF INCREASING AMOUNTS OF Ea AND VARIOUS DEAMINATED AND DENATURED DERIVATIVES TO 1.0 ML. OF ANTI-Ea SERUM FK2 1 → 4 AT 0°

| Ea N added, mg. | Total N pptd., mg. | Antibody N by diff., mg. | Antibody N: antigen N ratio | Supernatant tests | Prepn. added | Antigen N added, mg. | Total N pptd., mg. | Prepn. added | Antigen N added, mg. | Total N pptd., mg. |
|---|---|---|---|---|---|---|---|---|---|---|
| 0.010 | 0.209 | 0.199 | 19.9 | Excess antibody | 4B | 0.030 | 0.472 | 3A | 0.030 | 0.068 |
| .020 | .355 | .335 | 16.8 | Excess antibody | 4B | .051 | .588 | 3A | .050 | .086 |
| .030 | .466 | .436 | 14.5 | Excess antibody | 4B[a] | .075 | .644 | 4A | .027 | .050 |
| .060 | .640 | .580 | 9.7 | Excess antibody | 4B | .170 | .543 | 4A | .047 | .052 |
| .074[a] | .690 | .618 | 8.1 | | 4B | .500 | .135 | DnEa 101 | .030 | .086 |
| .080[b] | .694 | | | Trace antibody; 0.004 mg. Ea | 5B | .030 | .460 | DnEa 101 | .050 | .100 |
| .100 | .610 | | | Excess Ea | 5B | .050 | .606 | Ht Dn 27 | .034 | .064 |
| .174 | .304 | | | Excess Ea | 5B[a] | .075 | .676 | Ht Dn 27 | .068 | .092 |
| .464 | .055 | | | Excess Ea | 5B | .158 | .622 | | | |
| | | | | | 5B | .520 | .117 | | | |
| | | | | | 8B (–SH) | .032 | .482 | | | |
| | | | | | 8B (–SH) | .065 | .646 | | | |
| | | | | | 8B (–S–S–) | .030 | .464 | Similar values obtained with 5A, 6A, 8A (–S–S), 8A (–SH) | | |
| | | | | | 8B (–S–S–) | .060 | .640 | | | |
| | | | | | Similar values obtained with 3B and 6B | | | | | |

[a] Reaction run at 37° for 1 hr. [b] 0.076 mg. precipitated by antibody.

TABLE II

ADDITION OF INCREASING AMOUNTS OF B, EA AND A FRACTIONS TO 1.0 ML. OF ANTI-4B SERUM $979_1$ (1 → 10) AT 0°

| 4B N added, mg. | Total N pptd., mg. | Antibody N by diff., mg. | Antibody N: antigen N ratio | Supernatant tests | Prepn. added | Antigen N added, mg. | Total N pptd., mg. | Prepn. added | Antigen N added, mg. | Total N pptd., mg. |
|---|---|---|---|---|---|---|---|---|---|---|
| 0.0033 | 0.047 | 0.044 | 13.3 | Excess antibody | Ea | 0.0116 | 0.130 | 3A | 0.0093 | 0.019 |
| .005 | .067 | .062 | 12.4 | Excess antibody | Ea | .0174 | .162 | 3A | .016 | .017 |
| .010 | .104 | .094 | 9.4 | Excess antibody | Ea[b] | .023 | .169 | 4A | .009 | .017 |
| .015 | .142 | .127 | 8.5 | Excess antibody | Ea | .046 | .085 | 4A | .015 | .017 |
| .020[a] | .164 | .144 | 7.2 | No antibody or 4B | 3B | .0093 | .107 | 5A | .008 | .017 |
| .025 | .171 | .146 | | Excess 4B | 3B | .014 | .139 | 5A | .015 | .018 |
| .050 | .107 | | | Excess 4B | 5B | .0105 | .116 | | | |
| | | | | | 5B[b] | .020 | .163 | | | |
| | | | | | 5B | .050 | .107 | | | |

[a] Same values at 37°. [b] Run at 37°.

TABLE III

ADDITION OF INCREASING AMOUNTS OF ACID DnEa, Ea, AND ITS DEAMINATED AND DENATURED DERIVATIVES TO 1.0 ML. OF POOLED ANTI-ACID DnEa SERA $795_1$ AND $969_1$ (1 → 2) AT 37°

| DnEa N added, mg. | Total N pptd., mg. | Antibody N by diff., mg. | Antibody N: antigen N ratio | Supernatant tests | Prepn. added | Antigen N added, mg. | Total N pptd., mg. | Prepn. added | Antigen N added, mg. | Total N pptd., mg. |
|---|---|---|---|---|---|---|---|---|---|---|
| 0.0055 | 0.059 | 0.053 | 9.6 | Excess antibody | Ea | 0.070 | 0.002 | 4A | 0.021 | 0.063 |
| .023 | .088 | .065 | 2.8 | Excess antibody | Ea | .124 | .003 | 4A | .053 | .071 |
| .057 | .112 | | | Excess antibody, excess DnEa | 6B | .026 | .005 | 6A | .0216 | .070 |
| .086 | .116 | | | Excess DnEa | 6B | .069 | .006 | 6A | .065 | .079 |
| | | | | | 6B Dn | .022 | .086 | 6A Dn | .024 | .070 |
| The above points were obtained with DnEa lot 102. Selected points run with DnEa 105 and 106 were on the same curve. | | | | | 6B Dn | .066 | .095 | 6A Dn | .064 | .084 |
| | | | | | DnEa 105 Deam –SH | .042 | .094 | 8A | .025 | .062 |
| | | | | | DnEa 105 Deam –SH | .085 | .099 | 8A | .063 | .036 |
| | | | | | DnEa 105 Deam –S–S– | .0423 | .094 | 8A Dn | .025 | .069 |
| | | | | | DnEa 105 Deam –S–S– | .085 | .099 | 8A Dn | .064 | .055 |
| | | | | | Ea FNA | .069 | .074[a] | 8A FNA | .068 | .069[a] |

[a] Single determination only. 3A, 5A and 5A Dn behaved like 4A, 6A and 6A Dn. 8B Dn was similar to 6B Dn. DnEa 106 Deam –SH and –S–S– similar to the 105 preparations.

heat, acid and alkali were all closely related immunologically through the region of antibody excess and into the equivalence zone.[6] The type or the degree of aggregation appeared to have no influence on the results except in the region of antigen excess, where quantitative differences could be

TABLE IV

ADDITION OF INCREASING AMOUNTS OF A, Ea AND ITS DEAMINATED AND DENATURED DERIVATIVES TO 1.0 ML. OF ANTI-4A SERUM $990_1$ (1 → 2) AT 37°

| 4A N added, mg. | Total N pptd., mg. | Antibody N by diff., mg. | Antibody N: antigen N ratio | Supernatant tests | Prepn. added | Antigen N added, mg. | Total N pptd., mg. | Prepn. added | Antigen N added, mg. | Total N pptd., mg. |
|---|---|---|---|---|---|---|---|---|---|---|
| 0.0035 | 0.042 | 0.0385 | 11.0 | Excess antibody | Ea | 0.0046 | 0.004 | 5A | 0.009 | 0.091 |
| .0053 | .061 | .056 | 10.6 | Excess antibody | Ea | .0184 | .008 | 5A | .023 | .136 |
| .0106 | .092 | .081 | 7.6 | Excess antibody | 6B | .0068 | .007 | 8A | .0125 | .108 |
| .016 | .138 | .122 | 7.6 | Excess antibody | 6B | .0168 | .012 | 8A | .025 | .150 |
| .021 | .155 | .134 | 6.4 | Trace antibody; | 8B Dn | .012 | .051 | 8A Dn | .0125 | .100 |
| | | | | trace 4A | 8B Dn | .024 | .080 | 8A Dn | .025 | .146 |
| .027 | .147 | | | Excess 4A | DnEa 105 | .0086 | .040 | 8A FNA | .0076 | .066[a] |
| | | | | | DnEa 105 | .022 | .082 | 8A FNA | .023 | .144[a] |
| | | | | | | | | Ea FNA | .0075 | .068[a] |
| | | | | | | | | Ea FNA | .0225 | .143[a] |

[a] Single determination only.

observed between various DnEa preparations. The A fractions however, differed from DnEa in not precipitating as much antibody from anti-DnEa sera even in the region of antibody excess, while a still greater deficit occurred in the region of antigen excess. Similar results were obtained with cross reactions of acid DnEa with an anti-4A serum. The serological specificity of the ADn fractions in anti-4A and anti-acid DnEa sera differed little from that of A itself. Both Ea FNA and 8A FNA in their reduced (–SH) and oxidized (–S–S–) forms reacted serologically as normal A fractions.

The immunochemical results confirm the physical and chemical data showing that the denatured protein arrived at as a result of deamination is different from the other denatured preparations and so furnish another example of the utility of immunochemistry in the study of proteins.[13] The immunological evidence indicates not only that the processes characteristic of acid denaturation fail to reach completion in fraction A but also that the molecule may have unfolded into so stable a configuration that further acid treatment causes little or no change in structure. The new configuration arrived at during the unfolding of the protein molecule is much more resistant than Ea or fraction B to acid denaturation and is also more stable than acid DnEa to aggregating influences.

The BDn preparations had a smaller particle weight than acid DnEa[4] and reacted toward antisera like DnEa of a low degree of aggregation. Accordingly, BDn was indistinguishable from DnEa in the region of antibody excess in an anti-DnEa serum, while in the region of excess antigen BDn precipitated less total N than did DnEa. In anti-A serum BDn behaved like acid DnEa with no observable influence of the state of aggregation.

Both the –S–S– and –SH forms of deaminated DnEa showed definite differences from DnEa in anti-DnEa sera in that they precipitated somewhat less N. However, in anti-4A serum obtained after a second course of injections the DnEa Deam behaved like DnEa. Ea and fraction B cross reacted with anti-DnEa and anti-4A sera to a very slight degree.[14]

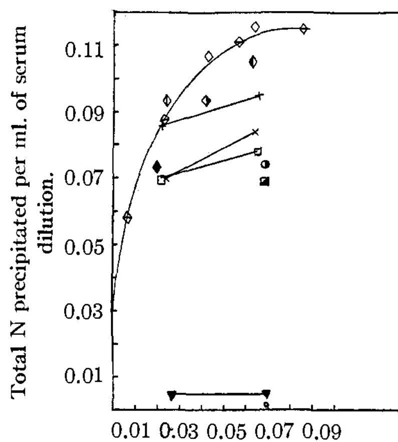


Fig. 3.—Homologous and cross precipitation in pooled anti acid DnEa sera $795_1$ and $969_1$ (1 → 2) Key to chart: ⊖, DnEa 101; ◇, DnEa 105; ◇, DnEa 105 Deam SH; ◇, DnEa 106; ◇, DnEa 106 Deam SH; ◆, DnEa 106 Deam SS; □, 6A; ×, 6A Dn; ▼, 6B; +, 6B Dn; O, EA; ◑, Ea FNA; ◪, 8A FNA.

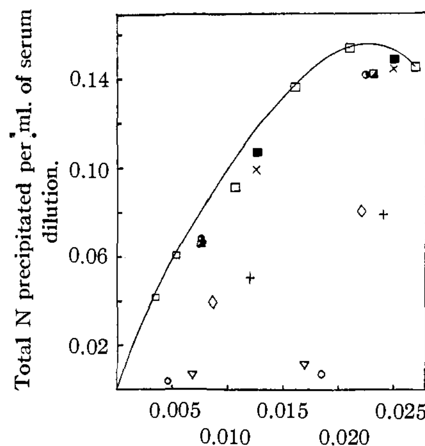


Fig. 4.—Homologous and cross precipitation with anti-4A serum $990_1$ (1 → 2): Key to chart: □, 4A; ■, 8A; ◪, 8A FNA; O, Ea; ◑, EA FNA; ◇, DnEa 105; +, 8B Dn; ×, 8A Dn; ▽, 6B.

(13) E. A. Kabat, *J. Immunol.*, **47**, 513 (1943).

(14) *Cf.* also C. F. C. MacPherson and M. Heidelberger, *Proc. Soc. Exptl. Biol. Med.*, **43**, 646 (1940).

The definite physical and chemical differences[4] as well as the antigenicity and characteristic specificity of the various denatured forms of Ea (also ref. 6) studied in this Laboratory show a reproducibility, degree of organization and specificity indicating that they have not reached the final step in the denatured state characterized as a completely random one[15] devoid of any specific structure. The present studies therefore corroborate the concept that various degrees of denaturation exist[16] and suggest that denaturation may take place by more than one pathway to yield different end products.

(15) W. T. Astbury, S. Dickinson and K. Bailey, *Biochem. J.*, **29**, 2351 (1935).

(16) H. P. Lundgren and J. W. Williams, *J. Phys. Chem.*, **43**, 989 (1939); H. P. Lundgren, *J. Biol. Chem.*, **138**, 293 (1941); H. Neurath, J. P. Greenstein, F. W. Putnam and J. O. Erickson, *Chem. Revs.*, **34**, 157 (1944).

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[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF HARVARD UNIVERSITY, DEPARTMENT OF SURGERY OF BETH ISRAEL HOSPITAL AND HARVARD MEDICAL SCHOOL]

# Synthesis of Naphthyl Carbonate Derivatives of Amino Acids as Chromogenic Substrates for Carboxypeptidase[1]

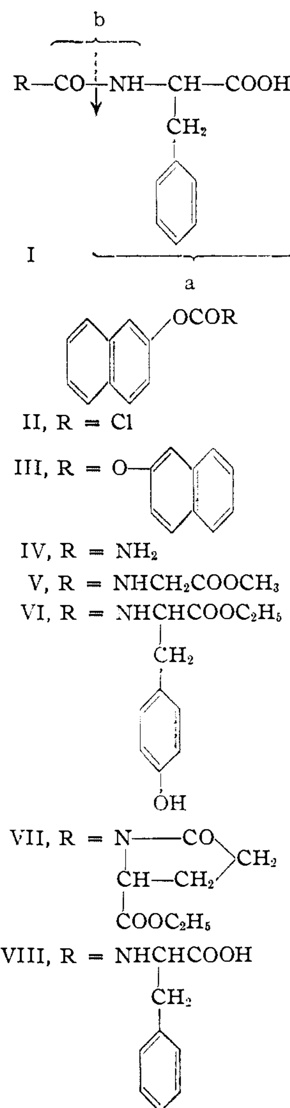
BY GEORGE WOLF AND ARNOLD M. SELIGMAN

The synthesis of carbonaphthoxyphenylalanine is described. Since the peptide is colorless, slightly soluble in aqueous solution, moderately stable at *p*H 7.8, unattacked by esterase and lipase (preliminary observation) and hydrolyzed by crystalline carboxypeptidase or by pancreatic tissue which has been subjected to tryptic digestion, it appears to possess properties on which to base a method for the colorimetric demonstration of carboxypeptidase.

The structural requirements of substrates susceptible to enzymatic hydrolysis by carboxypeptidase have been defined.[2] These are a phenylalanine or tyrosine residue with a free carboxyl group (Ia), a peptide link which is hydrolyzed by the enzyme (Ib), and acyl group (I; RCO), which may consist of a peptide chain, a benzoylglycyl group (I; R = $C_6H_5CONHCH_2$–), carbobenzoxy residue (I; R = $C_6H_5CH_2O$–), chloroacetyl group (I; R = $ClCH_2$–) or acetyl group (I; R = $CH_3$–).

In order to make possible the development of highly colored azo dyes from the hydrolysis product of the substrate for the colorimetric determination or histochemical demonstration of enzymatic activity, analogous compounds with a carbonaphthoxy group (I; R = $C_{10}H_7O$–) were prepared. Following enzymatic hydrolysis, naphthyl carbonate would be expected to lose $CO_2$ to form $\beta$-naphthol which could be converted to an azo dye as in methods developed previously for a variety of enzymes.[3–9]

On the basis of earlier studies[2] with synthetic substrates, it was expected that carbonaphthoxyphenylalanine (VIII) would provide sufficient specificity for demonstrating carboxypeptidase. In addition to the diester, dinaphthyl carbonate




(1) This investigation was supported by a research grant from the National Cancer Institute of the National Institutes of Health, Public Health Service, (in part) by a grant from the American Cancer Society (Massachusetts Division), and (in part) by an institutional grant to Harvard University from the American Cancer Society.

(2) H. Neurath and G. W. Schwert, *Chem. Revs.*, **46**, 69 (1950).

(3) M. L. Menten, J. Junge and M. H. Green, *J. Biol. Chem.*, **153**, 471 (1944).

(4) L. H. Manheimer and A. M. Seligman, *J. Nat. Cancer Inst.*, **9**, 181 (1948).

(5) M. M. Nachlas and A. M. Seligman, *ibid.*, **9**, 415 (1949).

(6) M. M. Nachlas and A. M. Seligman, *Anat. Record*, **105**, 677 (1949).

(7) M. M. Nachlas and A. M. Seligman, *J. Biol. Chem.*, **181**, 343 (1949).

(8) A. M. Seligman, M. M. Nachlas, L. H. Manheimer, O. M. Friedman and G. Wolf, *Ann. Surg.*, **130**, 333 (1949).

(9) A. M. Seligman and M. M. Nachlas, *J. Clin. Invest.*, **29**, 31 (1950).