Amino Acid Composition and Quaternary Structure of an Immobilizing Antigen from *Paramecium aurelia**

Edward Steers, Jr.

ABSTRACT: The immobilization antigen A isolated from strain 51 of *Paramecium aurelia* exists as a polymer of approximately 310,000 mw as determined by equilibrium centrifugation. Reduction with 2-mercaptoethanol results in a marked decrease in the native molecular weight to approximately 35,000. Dissociation of the native aggregate to a 35,000 molecular weight monomer is achieved only under conditions of reduction in the presence of urea or guanidine hydrochloride. The absence of any free sulfhydryl groups in the native molecule demonstrates the presence of interchain disulfide bonds. Amino acid analyses reveal 136 disulfide bridges/mole in the native molecule. The amino acid data together with previously reported fingerprint analyses for this protein indicate the presence of three sequentially different polypeptide chains.

The results of this study are consistent with a native polymer of 310,000 mw which is composed of nine monomeric units of 35,000 mw, of which three are of different primary sequence. These monomers are associated, in part at least, by disulfide bridges.

The exposure of live paramecia to homologous antiserum results in a progressive immobilization of the animals which ends in their death. This reaction is characterized by an agglutination of the tips of the cilia, rendering the animals immobile. Sonneborn (1948) was able to demonstrate that a given strain of *Paramecium aurelia* may exist in several different antigenic states (designated as serotypes). Although the total number of serotypes which any given strain of *Paramecium aurelia* is able to express is unknown, animals of one strain, strain 51, are capable of exhibiting at least ten different serotypes (Sonneborn, 1954). Individual cells of a particular strain, however, are only able to express one type of antigen at any given time.

Much of the original interest in the serotype system of Paramecium was concerned with the genetic phenomena involved in the expression of a particular serotype. Although the antigenic specificity of each of the serotypes is clearly under the control of a nuclear gene (Sonneborn and LeSeur, 1948; Sonneborn, 1950), the expression of each specificity locus is under the control of the cytoplasmic state of the animal (Sonneborn and LeSeur, 1948). The cytoplasmic state, in turn, is influenced by the environment. Thus, animals which are genotypically identical may appear phenotypically diverse. The mechanisms involved in the activation and repression of the specificity loci are unknown at present. The complex interactions of gene, cytoplasm, and environment involved in the expression of the serotype system in *Paramecium aurelia* has been extensively reviewed by Beale (1957).

Several reports have appeared in the literature which have been concerned with various biochemical characteristics of the specific substance responsible for the immobilizing reaction. Preer (1959a) has successfully isolated several of the immobilization antigens and has shown that they constitute the major water-soluble protein associated with the cilia. Preer (1959b) has further demonstrated that the molecule is a relatively large, fibrous protein, the molecular weight of which was estimated at approximately 240,000. This report is concerned with certain of the physicochemical properties of one of the immobilization antigens manifested by strain 51 of *Paramecium aurelia*, with particular reference to its amino acid composition and subunit structure.

Materials and Methods

Culture Methods and Purification. Strain 51, serotype A of variety 4 from *Paramecium aurelia* (isolated at Spencer, Ind.), was used throughout the course of this study. Isolation and culturing conditions of the clonal populations were identical with those previously described by Steers (1962). This procedure has consistently yielded mass cultures of 320 liters containing population levels of between 2000 and 3000 animals/ml.

Purification of the immobilization antigen 51A was carried out according to the procedure of Preer (1959a). A single lot of paramecia (300–400 liters) generally yielded an average of 35 to 40 mg of purified immobilization antigen.

Amino Acid Analyses. Amino acid analyses were carried out on aliquots containing approximately 2 mg of protein each. The lyophilized material was suspended in 5.7 N constant-boiling HCl and equal aliquots were transferred to thick-walled hydrolysis tubes. The samples were thoroughly evacuated, sealed,

* From the Laboratory of Chemical Biology, National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Md. *Received May 3, 1965.*

TABLE I: Amino Acid Composition of Immobilization Antigen 51 A.

Amino Acid	Extrapolated Value	Hours Hydrolyzed 22	44	66	88	μmole %[a]	Residues per 310,000
Lysine	0.166	0.166	0.165	0.166	0.166	5.09	148
Histidine	0.023	0.023	0.024	0.023	0.022	0.70	20
Arginine	0.043	0.043	0.042	0.042	0.045	1.31	38
Aspartic acid	0.383	0.370	0.377	0.383	0.384	11.76	343
Threonine[b]	0.488	0.478	0.465	0.455	0.452	15.00	438
Serine[b]	0.275	0.268	0.254	0.240	0.236	8.44	246
Glutamic acid[c]	0.200	0.184	0.198	0.202	0.200	6.15	179
Proline	0.078	0.077	0.075	0.090	0.067	2.37	69
Glycine	0.239	0.234	0.235	0.239	0.248	7.35	214
Alanine[c]	0.399	0.384	0.390	0.397	0.402	12.31	359
Half-cystine	0.304	0.302	0.316	0.300	0.304	9.34	272
Valine[c]	0.163	0.154	0.154	0.159	0.166	4.99	146
Methionine[b]	0.014	0.014	0.013	0.013	0.005	0.41	12
Isoleucine	0.092	0.089	0.090	0.091	0.093	2.82	82
Leucine	0.131	0.129	0.127	0.128	0.133	4.01	117
Tyrosine	0.091	0.091	0.089	0.093	0.094	2.81	82
Phenylalanine	0.052	0.053	0.049	0.054	0.053	1.65	48
Tryptophan[d]						3.50	101
Totals						100.02	2914

[a] Expressed as per cent of total micromoles recovered from chromatography. [b] Value obtained by linear extrapolation to zero time. [c] Average value from the 44–88 hour hydrolyses. [d] Value taken from Preer (1959b).

and subjected to hydrolysis at 110° for periods of 22, 44, 66, and 88 hours. At the end of the appropriate time interval the tubes were opened and the contents were evaporated to dryness *in vacuo* over NaOH. The dried hydrolysates were then subjected to ion-exchange chromatography according to the techniques of Moore and Stein (1954) utilizing the Beckman Model 120 automatic amino acid analyzer.

Reduction and Alkylation. The procedure for reducing and alkylating the disulfide bridges present in the native molecule was carried out by the method of Anfinsen and Haber (1961). The protein (40 mg) was dissolved in 20 ml of 8 M urea (twice recrystallized from ethanol) at *p*H 8.0. 2-Mercaptoethanol (Eastman Organic) was added to a final concentration of 0.1 M, and the flask was tightly stoppered and incubated for 4 to 6 hours at 38°. Following reduction, the solution was diluted with distilled water to a final concentration of approximately 2 M with respect to urea. Recrystallized iodoacetic acid was then added to a final concentration of 10 mg/mg of protein. The alkylation was allowed to proceed at room temperature for 20 minutes, during which time the *p*H was maintained at 8.0–8.5 with NaOH. The alkylation was terminated by the addition of an excess of 2-mercaptoethanol to remove unreacted iodoacetic acid. The reduced-carboxymethylated antigen (RCMA)[1] was dialyzed against 0.05 M Tris buffer, *p*H 7.5, in which it was readily soluble at concentrations up to 10 mg/ml.

Ultracentrifugation. A Spinco Model E analytical ultracentrifuge equipped with the standard schlieren and Raleigh optical systems was employed. Temperature was controlled at 20° on all runs with the RTIC temperature control unit. Sedimentation values were calculated as described by Schachman (1957), and corrected to water, 20° at zero protein concentration, except where indicated.

For speeds less than 17,000 rpm, the special AN-J rotor was used. For all centrifugations above 17,000, the standard AN-D rotor was used.

High-speed equilibrium centrifugation was carried out by the technique of Yphantis (1964), utilizing the specially designed six-channeled centerpiece. All equilibrium measurements were made between 22 and 30 hours of centrifugation.

A partial specific volume of 0.71, determined by Preer (1959b), was used for all molecular weight determinations. The ultracentrifugal analyses were carried out in a Tris buffer unless indicated otherwise. Tris buffer consisted of 0.05 M Tris-HCl, *p*H 7.5, 0.1 M NaCl, and 0.01 M $MgCl_2$.

Results

Amino Acid Composition. The results of the amino

[1] Abbreviations used in this work: RCMA, reduced-carboxymethylated antigen; CMC, carboxymethyl-cysteine; SDS, sodium dodecyl sulfate; βSH, 2-mercaptoethanol; G-HCl, guanidine hydrochloride.

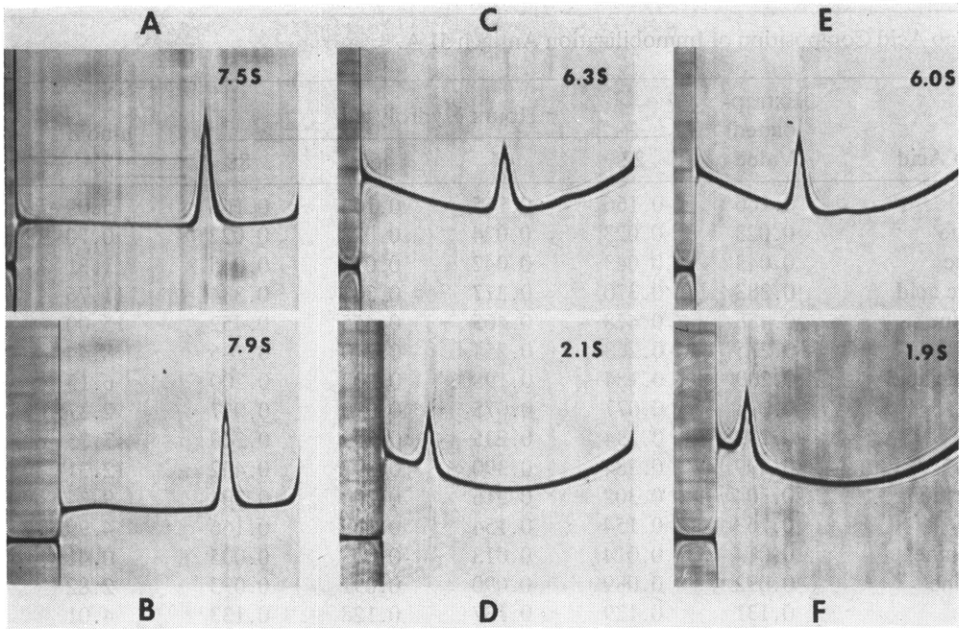

FIGURE 1: Results of a series of ultracentrifugal experiments on the effect of various dissociating conditions.

TABLE II: Results of Alkylation Under Various Conditions.

Alkylating Conditions[a]	Micromoles Half-cystine	CMC[c]	Total
Tris buffer	0.302	0	0.302
8 M urea	0.295	0	0.295
6 M G-HCl	0.310	0	0.310
8 M Urea, βSH	0.020	0.277	0.297
6 M G-HCl, βSH	0.013	0.280	0.293
2 × 8 M Urea, βSH[b]	0.013	0.300	0.313

[a] All alkylations were carried out in a buffer consisting of 0.05 M Tris-HCl, 0.1 M NaCl, *p*H 8.0, at room temperature for 20 minutes. Reduction was carried out with 2-mercaptoethanol (βSH) at a concentration of 0.1 M as described in Materials and Methods. [b] Sample subjected to a second reduction and alkylation. [c] Carboxymethyl-cysteine.

acid analyses are presented in Table I. The data are expressed in μmoles for each amino acid recovered from chromatography. The zero-time values are obtained by linear extrapolation from the 22 to 88 hour hydrolyses except for valine, alanine, and glutamic acid, which appear to increase significantly after 44 hours. The zero-time values for these three amino acids are calculated by averaging the values obtained from the 44- to 88-hour hydrolysates. No apparent loss of tyrosine is evident with time, while serine and threonine show a characteristic time-dependent destruction. The methionine content shows a marked decrease only after 66 hours of hydrolysis. The value for tryptophan is that reported by Preer (1959b) which was determined spectrophotometrically by the method of Goodwin and Morton (1946). Column 7 of Table I reports the content of each amino acid as the fraction of total μmoles recovered from chromatography. Column 8 lists the number of residues to the nearest integer per mole, assuming a molecular weight of 310,000.

Sulfhydryl Content. Table II summarizes the results of a series of experiments attempting to determine the true nature of the half-cystine residues resulting from acid hydrolysis. The native protein was alkylated under a variety of denaturing conditions with and without prior reduction by 2-mercaptoethanol. The complete absence of any carboxymethyl-cysteine (CMC) in those samples which were alkylated in the absence of 2-mercaptoethanol in either urea or guanidine hydrochloride indicates the absence of free sulfhydryl groups in the native protein. In contrast, the analyses on reduced-carboxymethylated samples always resulted in the presence of small amounts of half-cystine (3–6%). The high percentage of carboxymethyl-cysteine (averaging between 94 and 97% of the total half-cystine-CMC values) obtained after standard reduction with 2-mercaptoethanol clearly indicates that conditions which rupture disulfide bridges result in the subsequent alkylation of nearly all the sulfhydryl groups. All attempts to obtain complete recovery of carboxymethyl-cysteine upon chromatography were ineffective. Half-cystine values in the range of 3–6% of the total half-cystine-CMC values were consistently obtained after acid hydrolysis. As seen in Table II, subjecting the reduced carboxymethylated derivative to a second reduction and alkylation did not result in further conversion of the

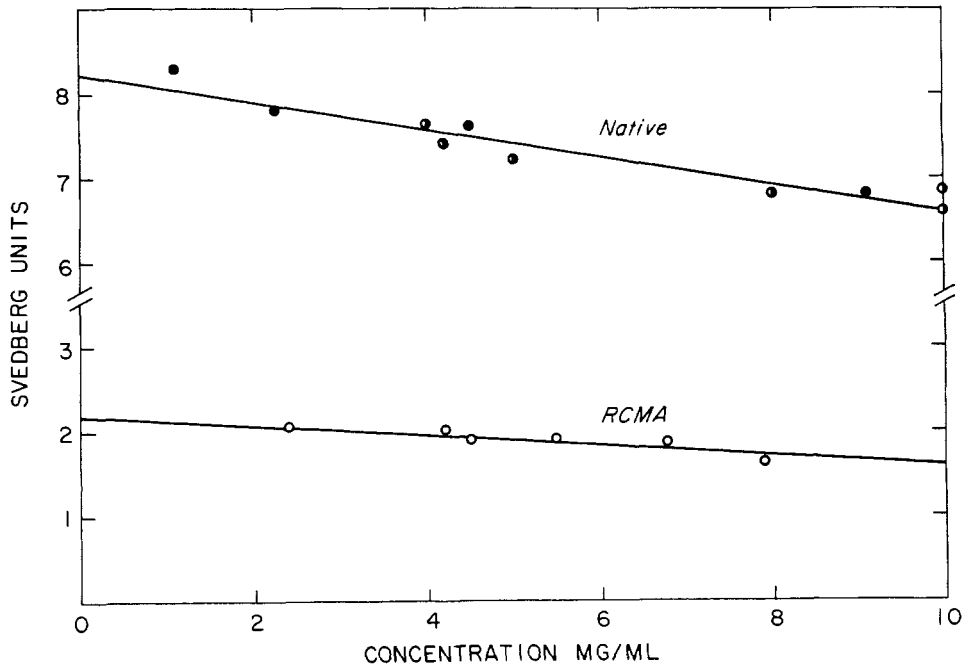

FIGURE 2: Concentration dependence of the $s_{20,w}$ for the native protein and the fully reduced carboxymethylated derivative.

half-cystine value to carboxymethyl-cysteine. The conversion of small amounts of CMC by acid hydrolysis to half-cystine (in the range of 5%) as detected by subsequent chromatography has been reported by several investigators (Fraenkel-Conrat *et al.*, 1951; Lindley, 1955; Sela *et al.*, 1959). Reduction and subsequent alkylation of the native protein in dodecyl sulfate (SDS) have not been possible. The addition of reducing amounts of 2-mercaptoethanol to the protein-SDS solutions results in the formation, within a few minutes, of a heavy precipitate which remains insoluble in SDS.

Sedimentation-Velocity Experiments. Figure 1 summarizes the results of a series of ultracentrifugal experiments on the effect of the dissociating conditions listed in Table II upon the immobilization antigen. Figure 2 demonstrates the concentration dependence of the $s_{20,w}$ for the native protein and the fully reduced-carboxymethylated derivative. A sedimentation coefficient of $s^0_{20,w} = 8.3$ for the native protein was calculated, which is in complete agreement with the value reported by Preer (1959b). The reduced-carboxymethylated antigen was determined to have a sedimentation coefficient of $s^0_{20,w} = 2.2$. Frame 1A of Figure 1 illustrates the sedimentation pattern for the native protein in Tris buffer. Frame 1B represents the same conditions except that dodecyl sulfate was added to a final concentration of 0.5%. Frames 1C and 1E represent the native protein in 8 M urea and 6 M guanidine hydrochloride, respectively. Frames 1D and 1F represent the same samples as frames 1C and 1E, with the addition of reducing amounts of 2-mercaptoethanol. All sedimentation values appearing in Figure 1 are corrected to water at 20° but are not corrected to zero protein concentration. The effect of 2-mercaptoethanol in dissociating solvents such as those employed here is a marked decrease in the sedimentation value. There appears to be no dissociating effect of urea, guanidine hydrochloride, or dodecyl sulfate in the absence of reducing conditions (2-mercaptoethanol).

Molecular Weight Determinations. Table III presents the results of a series of high speed equilibrium centrifugation experiments with the native antigen dissolved in Tris buffer, *p*H 7.5; in 8 M urea, *p*H 7.5; and with

TABLE III: Molecular Weight Determinations.[a]

Conditions	0.3 mg/ml	0.1 mg/ml
Native	281,000	295,000
Tris buffer	329,000	
	327,000	319,000
	319,000	
	318,000	294,000
Native		338,000
8 M urea		347,000
RCMA		30,000
Tris buffer		34,000
		38,000
		39,000

[a] All runs employed 3-mm columns of solution at speeds in the range of 10,589 to 12,590 rpm except for the RCMA samples which were run at speeds in the range of 31,410 to 35,600 rpm.

the reduced-carboxymethylated derivative dissolved in Tris buffer, *p*H 7.5. The average molecular weight calculated from these eight experiments is 310,000 ± 20,000 for the native molecule at neutral *p*H. Two determinations were made on the native molecule in 8 M urea to gain an indication of whether or not this relatively strong dissociating solvent resulted in any change in molecular weight. As mentioned above, there was only a slight decrease in the sedimentation coefficient under these conditions, indicating that no dissociation of the molecule had occurred ($s = 7.5$ to $s = 6.3$). The two weight determinations are in reasonably close agreement with each other but significantly higher than the molecular weight in Tris buffer: 342,000 and 310,000, respectively. The molecular weight determination for the reduced-carboxymethylated derivative in Tris buffer, however, is 35,000, reflecting a decrease in the molecular weight by a factor of approximately nine.

Discussion

The molecular weight of the native molecule is calculated to be 310,000. This value is significantly higher than that reported by Preer (1959b) of 240,000 for 51 A, which was determined from sedimentation and diffusion measurements. The value of 240,000 is in agreement with a value of 250,000 subsequently reported by Bishop (1961) and Jones,[2] as determined by the Archibald approach to equilibrium method. In the Archibald determinations, no correction is reported for the effect of the protein concentration upon molecular weight. The finding by Preer (1959b) of a marked concentration dependence of the sedimentation constant, with little or no concentration effect on the diffusion constant, means that the sedimentation to diffusion ratio will vary with concentration. Such variation would suggest a dependence of molecular weight upon concentration as determined by the Archibald method. This conclusion is further strengthened by the fact that the native molecule is rather fibrous, with an axial ratio of 12:1 (Preer, 1959b). Such a dependency of molecular weight upon protein concentration as determined by the Archibald method has been demonstrated for other systems (Hofman and Harrison, 1963; Steers *et al.*, 1965). The original value of 240,000, reported by Preer, calculated from sedimentation and diffusion measurements, is subject to an error of 20% (Preer, 1959b) due to a variation of that magnitude in the calculated diffusion coefficient. For the purpose of discussion in this paper a molecular weight of 310,000 is assumed.

The molecular weight value of 340,000 for the native molecule in 8 M urea is somewhat higher than that of the native molecule in aqueous solution. The basis for this apparent discrepancy may be the fact that no difference in the partial specific volume was assumed in urea, an assumption that may not be justified. The significant finding in these determinations, however, is that the molecular weight of the protein in 8 M urea does not decrease, indicating the absence of dissociation.

The results of the equilibrium experiments on the reduced-carboxymethylated derivative are in marked contrast to the results presented above. Reduction and alkylation results in a derivative with an apparent molecular weight of 35,000. These results indicate the presence of approximately nine polypeptide chains/mole in the native molecule.

The absence of any apparent dissociation of the native molecule in dodecyl sulfate, urea, or guanidine hydrochloride solutions indicates that the subunits of the native molecule are associated by covalent forces. The dissociation of these subunits occurs only under reducing conditions, demonstrating that these interchain covalent linkages are disulfide bonds. These results, however, do not differentiate between disulfide bonds which exist as interchain bonds and those which may exist as intrachain bonds.

The complete absence of carboxymethyl-cysteine following the standard conditions of alkylation reflects the absence of any free sulfhydryl groups. The number of half-cystine residues (272) reflects a total of 136 disulfide bridges/mole in the native protein. This represents approximately one cystinyl residue for every 20 amino acids.

An indication of whether any, if not all, of the subunit polypeptide chains are identical in their primary sequence may be derived from the amino acid data of Table I and the analysis of tryptic digests by peptide mapping reported previously (Steers, 1962, 1963). On the basis of the native molecular weight of 310,000, one expects the presence of 148 lysine residues and 38 arginine residues per mole. Following tryptic hydrolysis, a total of 187 tryptic peptides are expected. Upon fingerprinting the trypsin digest, a total of 66 ninhydrin-positive spots were detectable, indicating approximately 65 trypsin-sensitive bonds, or one-third of the number expected. Such peptide analysis allows one to calculate a minimum molecular weight of approximately 106,000 of unique primary sequence. As these patterns reflected only part of the tryptic hydrolysate due to the presence of some immobile "core" material (Steers, 1962), the number of observed tryptides must reflect a minimum number. A more accurate estimate of the minimum chemical molecular weight is made possible by detecting only the arginine-containing tryptides with the specific Sakaguchi stain. This analysis is possible because the immobile "core" is Sakaguchi negative, allowing one to conclude that all of the arginine tryptides were visualized in the fingerprint patterns. A total of 13 Sakaguchi-positive spots were detected. This, again, is approximately one-third of the total number expected (38) per molecular weight of 310,000. On the basis of the arginine content, a minimum chemical molecular weight of approximately 104,000 may be calculated.

The results of the current study are consistent with

[2] I. G. Jones, manuscript in preparation. Dr. Jones, in a comparable study, has also found evidence for nonidentical subunits which are associated through disulfide bonds in a different variety (2) of *P. aurelia*.

the conclusion that the native molecule consists of nine polypeptide chains of approximately 35,000 molecular weight, of which three are of different primary sequence. This model is based on a native molecular weight of 310,000, with the smallest identical subunit being on the order of 105,000. On the basis of a native molecular weight of 240,000, the molecule would be expected to contain only two identical subunits. The significant point of this report is the fact that the native molecule represents a polymer consisting of non-identical polypeptide chains, on the order of 35,000 mw, which are held together through disulfide bridges.

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References

Anfinsen, C. B., and Haber, E. (1961), *J. Biol. Chem. 236*, 1361.

Beale, G. H. (1957), *Intern. Rev. Cytol. 6*, 1.

Bishop, J. O. (1961), *Biochim. Biophys. Acta 50*, 471.

Fraenkel-Conrat, H., Mohammad, A., Ducay, E. D., and Mecham, D. K. (1951), *J. Am. Chem. Soc. 73*, 625.

Goodwin, T. W., and Morton, R. A. (1946), *Biochem. J. 40*, 628.

Hofman, T., and Harrison, P. N. (1963), *J. Mol. Biol. 6*, 256.

Lindley, H. (1955), *J. Am. Chem. Soc. 77*, 4927.

Moore, S., and Stein, W. H. (1954), *J. Biol. Chem. 21*, 907.

Preer, J. R., Jr. (1959a), *J. Immunol. 83*, 276.

Preer, J. R., Jr. (1959b), *J. Immunol. 83*, 378.

Schachman, H. (1957), *Methods Enzymol. 4*, 32.

Sela, M., White, F. H., and Anfinsen, C. B. (1959), *Biochim. Biophys. Acta 31*, 417.

Sonneborn, T. M. (1948), *Proc. Natl. Acad. Sci. U.S. 34*, 413.

Sonneborn, T. M. (1950), *Heredity 4*, 11.

Sonneborn, T. M. (1954), *Proc. Natl. Cancer Conference, 2nd 1954*, 1139.

Sonneborn, T. M., and LeSeur, A. (1948), *Am. Naturalist 82*, 69.

Steers, E., Jr. (1962), *Proc. Natl. Acad. Sci. U.S. 48*, 867.

Steers, E., Jr. (1963), PhD. dissertation, University of Pennsylvania.

Steers, E., Craven, G. R., Anfinsen, C. B., and Bethune, L. (1965), *J. Biol. Chem. 240*, 2468.

Yphantis, D. A. (1964), *Biochemistry 3*, 297.