[CONTRIBUTION OF THE BIOCHEMISTRY DEPARTMENT, UNIVERSITY OF WISCONSIN]

The Preparation and Physical Properties of Polypeptidyl Proteins[1,2]

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The polymerization of N-carboxyamino acid anhydrides and the modification of proteins by reaction with N-carboxyamino acid anhydrides in aqueous buffered solutions were improved by conducting the reactions in bicarbonate rather than in phosphate buffer. New polypeptidyl proteins were prepared by the addition of unsubstituted lysine by reaction with N-carboxy-L-lysine anhydride hydrobromide and by the addition of unsubstituted glutamic acid by reaction with N-carboxy-L-glutamic acid anhydride. These proteins modified with basic and acidic amino acids showed no appreciable change in their solubility. Electrophoresis experiments revealed a large change in their mobilities and in the case of glutamic acid modified protein the optical patterns suggested a homogeneous product.

Recently a number of polypeptidyl proteins have been prepared by the addition of amino acid polymers to proteins by reaction with N-carboxyamino acid anhydrides in aqueous buffered solutions.[3-7] Becker and Stahmann first prepared polyglycyl-bovine plasma albumin and polyglycyl-chymotrypsin. Green and Stahmann[5] prepared poly-γ-ethylglutamyl-bovine plasma albumin by the same method. The molecular weights of the proteins were increased from 12 to 18% by the addition of the amino acid polymers. In spite of this, the modified proteins showed no significant change in solubility and in the case of polyglycyl-chymotrypsin, the esterase activity of the protein was not measurably altered.[4] Tobacco mosaic virus has also been modified with C^{14}-labeled N-carboxyleucine anhydride[6] and with N-carboxyleucine, glycine and γ-ethylglutamic acid anhydrides[8] without appreciable change in the infectivity of the virus. Preliminary immunochemical studies showed that polyglycyl-bovine serum albumin can produce a characteristic antibody response and the results of the immunochemical studies showed that the modified protein can be distinguished from the native protein.[9] This communication deals with the improvements in the general method for treating N-carboxyamino acid anhydrides with proteins and the preparation of proteins modified by the attachment of basic and acidic amino acids. These are of particular value as a synthetic model to studies concerned with the physical, biochemical and immunochemical properties of proteins as they relate to the amino acid composition.

In polymerization of N-carboxyamino acid anhydrides in aqueous buffered solutions, the type of buffer anions has a most pronounced effect on the extent of polymerization.[10] For instance, with N-carboxyglycine anhydride between the *p*H range of 6 to 7, phosphate buffer gave about 80% polymerization and 20% hydrolysis, cacodylate, only 5% polymerization and 95% hydrolysis.[10] Arsenate, citrate and maleate buffers all gave values intermediary between the two extremes. However, the earlier studies on the effect of buffer anions on the polymerization of N-carboxy anhydrides were complicated because the bicarbonate ion from the CO_2 produced in the reaction could also act as a second buffer anion. To show the effect of a single species of buffer anion, bicarbonate was employed. The results of the polymerization of N-carboxyleucine anhydride in various buffers at the same *p*H showed that in the presence of bicarbonate ion polymerization occurred quantitatively and no hydrolysis to the parent amino acid could be measured. Phosphate gave 81% polymerization and 19% hydrolysis, while cacodylate gave 19 and 81%, respectively. This effect of bicarbonate buffer in minimizing the hydrolytic reaction was further demonstrated when N-carboxyamino acid anhydrides were treated with bovine plasma albumin in bicarbonate buffer. A much greater percentage of the anhydride reacted with the protein as shown in Table I. In the case of polyglycyl-bovine plasma al-

TABLE I

EFFECT OF BUFFER ANIONS ON THE REACTION OF N-CARBOXYAMINO ACID ANHYDRIDES WITH PROTEINS

Protein	Anhydride	Reacted, %	Moles amino acid added per mole protein	Molecular weight[d]
	Phosphate buffer			
BPA[a]	Glycine	3–27[e]	171	77,600
Chymotrypsin[b]	Glycine	26[e]	66	39,200
Chymotrypsin[b]	L-Leucine	2.4[f]	52.5	32,900
BPA	γ-Et-glutamic	25[g]	86	82,500
	Bicarbonate buffer			
BPA	L-Leucine	55–60	49	73,300
RSA[c]	L-Leucine	80–90	...	....
RSA[c]	DL-Phenylalanine	69	...	....
BPA	DL-Phenylalanine	83.5	48	76,000

[a] BPA = bovine plasma albumin, mol. wt., 69,000. [b] Mol. wt., 27,000. [c] RSA = rabbit serum albumin. [d] Calculated from amino acid added. [e] Calculated from results of Becker and Stahmann.[4] [f] Calculated from results of Becker.[11] [g] Calculated from results of Green and Stahmann.[5]

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(2) Presented before the Division of Biological Chemistry, American Chemical Society, Cincinnati, Ohio, April 1, 1955.

(3) M. A. Stahmann and R. R. Becker, THIS JOURNAL, **74**, 2695 (1952).

(4) R. R. Becker and M. A. Stahmann, *J. Biol. Chem.*, **204**, 745 (1953).

(5) M. Green and M. A. Stahmann, *ibid.*, **213**, 259 (1955).

(6) H. Fraenkel-Conrat, *Biochem. Biophys. Acta*, **10**, 180 (1953).

(7) M. Sela, *Bull. Res. Council Israel*, **4**, 109 (1954).

(8) H. Tsuyuki, Ph.D. Thesis, University of Wisconsin, 1955.

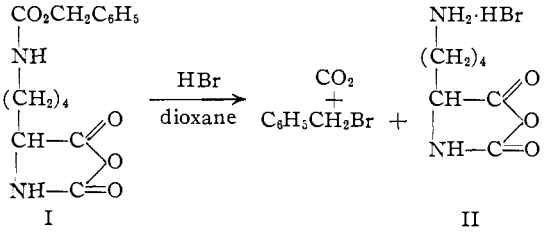
(9) T. Makinodan, R. R. Becker, H. R. Wolfe and M. A. Stahmann, *J. Immunol.*, **73**, 159 (1954).

(10) R. R. Becker and M. A. Stahmann, *J. Biol. Chem.*, **204**, 737 (1953).

(11) R. R. Becker, Ph.D. Thesis, Univ. Wisconsin, 1952.

bumin, polyglycylchymotrypsin[3,4] and poly-γ-ethylglutamyl-bovine plasma albumin,[5] a maximum of about 25% of the original anhydride was found attached to the protein as polypeptides when 0.07 *M*, *p*H 7.4, phosphate buffer was employed as the reaction medium. The remainder of the anhydride was lost either through hydrolysis or by the formation of insoluble polypeptides not attached to the proteins. In contrast, the use of bicarbonate buffer in the same type of reaction led to the addition of from 55 to 90% of the leucine and 69 to 84% of the DL-phenylalanine anhydrides to the proteins. This shows that the side reactions involved were reduced by the bicarbonate medium. In this improved method for the modification of proteins, only about one-fifth the amount of anhydride is required to prepare proteins modified to the same extent.

In the past it has been possible to modify proteins only with neutral amino acids[3,4,6] which could be added to the protein in the unsubstituted form or with γ-ethylglutamic acid[5] and O-carbobenzoxytyrosine.[7] A basic amino acid such as lysine could be added to the protein only through reaction with ε-carbobenzoxy-N-carboxylysine anhydride. The carbobenzoxy blocking group cannot be removed from the modified protein without employing drastic conditions such as treatment with phosphonium iodide[12] or with hydrogen bromide[13] in glacial acetic acid. Such reagents denature the protein or cause other undesirable alterations of the protein molecules. This difficulty was circumvented by the removal of the ε-carbobenzoxy group of ε-carbobenzoxy-N-carboxylysine anhydride (I) with hydrogen bromide in dioxane before reaction of the anhydride with the protein as shown in the following reaction scheme



I II

The anhydride II which separated as a sticky white precipitate was then treated with the protein in bicarbonate buffer in the usual manner. The polylysyl-protein showed a 23% increase in lysine content or 14 moles of lysine were added per mole of bovine plasma albumin (Table II).

Bovine plasma albumin has been modified by addition of glutamic acid by treating a 1% solution of protein in phosphate buffer with γ-ethyl-N-carboxyglutamic acid anhydride, followed by alkaline hydrolysis of the γ-ethyl ester group.[5] The alkaline treatment of both the unmodified and modified bovine plasma albumin under these conditions altered the protein in such a manner that their electrophoretic patterns were no longer symmetrical.[5] Consequently, in the present communication polyglutamyl-bovine plasma albumin was prepared by

(12) C. R. Harington and T. H. Mead, *Biochem. J.*, **29**, 1603 (1935).

(13) D. Ben-Ishai and A. Berger, *J. Org. Chem.*, **17**, 1564 (1952).

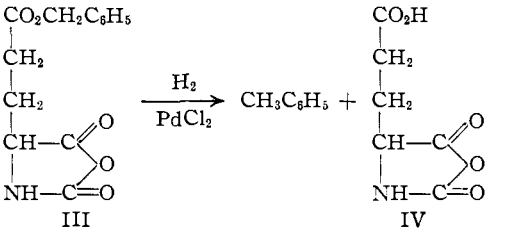
TABLE II

POLYLYSYL- AND POLYGLUTAMYL-ALBUMINS

Protein	Moles amino acid per mole BPA[a] Lysine	Glutamic	Moles amino acid added per mole BPA Lysine	Glutamic	Molecular weight
BPA	61[b]	77[b]	..	..	69,000
Polylysyl-BPA	75	77	14	..	70,800[c]
Polyglutamyl-BPA	61	350	..	273	104,200[c]

[a] BPA = bovine plasma albumin. [b] Calculated from W. H. Stein and S. Moore.[14] [c] Calculated from amino acid added.

reaction of the protein with N-carboxyglutamic acid anhydride (IV) in bicarbonate buffer. The N-carboxyglutamic acid anhydride (IV) was prepared by catalytic reduction of γ-benzyl-N-carboxyglutamic acid anhydride (III) in the presence of palladized charcoal as shown in the reaction scheme



III IV

The glutamic acid content increased from 77 moles per mole of bovine plasma albumin to 350 moles per mole of polyglutamyl-bovine plasma albumin. Molecular weight of the polyglutamyl-bovine plasma albumin calculated from the amount of glutamic acid added showed an increase of 35,200 over the unmodified control of 69,000 (Table II). In the modification of proteins by reaction with N-carboxyanhydrides of neutral amino acids and γ-ethylglutamic acid the extent of addition of the amino acid is limited by the insolubility of the polypeptidyl proteins in aqueous solutions. In the new method of modifying proteins by reaction with N-carboxyglutamic acid anhydrides the product is always soluble in the bicarbonate reaction medium and consequently a much larger increase in molecular weight can be achieved.

Electrophoresis was used to estimate the homogeneity and to determine the effect of the addition of lysine or glutamic acid residues to the protein on its electrophoretic mobility.[15] These electrophoretic experiments have provided additional evidence that the added amino acids and polypeptides are attached to the protein by chemical bonds rather than by an ionic interaction. An ionic interaction between polylysine and bovine plasma albumin would be indicated by separate polylysine and bovine plasma albumin boundaries in the two limbs of the electrophoresis cell and greatly changed mobilities for the descending components on each limb due to interaction.[16] In contrast, the polylysyl- and polyglutamyl-proteins both migrated as single boundaries and their behaviors in both limbs were similar,

(14) W. H. Stein and S. Moore, *Cold Spring Harbor Symposia, Quant. Biol.*, **14**, 179 (1950).

(15) H. Van Kley, M.S. Thesis, University of Wisconsin, 1955.

(16) R. V. Rice, M. A. Stahmann and R. A. Alberty, *J. Biol. Chem.*, **209**, 105 (1954).

thus providing further evidence that the mode of attachment of the polypeptides is through chemical bonds.

Polylysyl-bovine plasma albumin migrated as a single peak with a long tailing edge in *p*H 7.5 tris-(hydroxymethyl)-aminomethane hydrochloride buffer (Fig. 1a). The maximum gradient of the

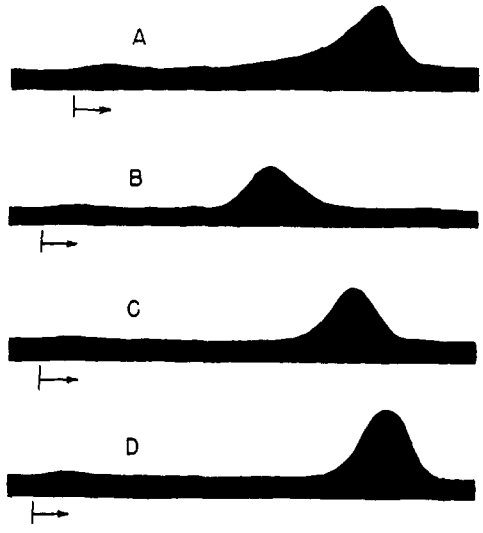


Fig. 1.—Descending electrophoretic patterns for polypeptidylbovine plasma albumin (BPA): A, polylysyl-BPA at 233 minutes (*p*H 7.5); B, polyglutamyl-BPA at 158 minutes (*p*H 5.3); C, polyglutamyl-BPA at 310 minutes (*p*H 6.3); D, BPA at 271 minutes (*p*H 7.5).

boundary curve showed no change in mobility compared to unmodified bovine plasma albumin. But when the average mobility was calculated by the first moment of the curve[17] a 10% decrease in mobility was found. These results indicate that the main portion of the protein was unmodified and that the N-carboxy-L-lysine anhydride hydrobromide did not react with every protein molecule. The initially modified albumin molecules contain more nucleophilic amino groups on their surface by introduction of the ε-amino groups of lysine and subsequent reaction of the anhydride with them is favored. Rabbit serum albumin treated with five separate portions of N-carboxy-L-lysine anhydride hydrobromide gave a non-uniform product as indicated by a long tail in the electrophoretic pattern, but the mobility of the entire preparation was changed. The calculated mobility values are given in Table III.

The electrophoretic behavior of polyglutamyl-bovine plasma albumin was investigated in *p*H 5.3 sodium acetate buffer (Fig. 1b) and *p*H 6.3 sodium cacodylate buffer (Fig. 1c). At these *p*H values the unmodified albumin has a net negative charge. The increase in the net negative charge by the addition of glutamic acid residues is expressed by an increase in electrophoretic mobility. The boundary pattern at *p*H 5.3 showed a slight asymmetry and an average mobility increase of 6.80 mobility units or a 324% increase. An essentially symmetrical boundary was observed at *p*H 6.3; the mobility increase in this case was 7.28 mobility units or a

(17) R. A. Alberty, *J. Chem. Ed.*, **25**, 426 (1948).

TABLE III

ELECTROPHORETIC MOBILITIES OF POLYPEPTIDYL PROTEINS

Protein	Buffer	*p*H	Mobility × 10^5 cm.^{2} v.$^{-1}$ sec.$^{-1}$	Change in mobility × 10^5	Change in mobility %
BPA[a]	Tris-chloride[e]	7.5	−4.80	...	..
BPA	Sodium cacodylate	6.3	−2.51	...	..
BPA	Sodium acetate	5.3	−2.10	...	..
RSA[b]	Sodium acetate	5.3	−1.68	...	..
Polylysyl-BPA	Tris-chloride[e]	7.5	−4.79[c]	0	0
			−4.32[d]	−0.58	10
Polylysyl-RSA	Sodium acetate	5.3	−1.34[c]	−0.29	18
			−1.13[d]	−0.50	31
Polyglutamyl-BPA	Sodium acetate	5.3	−8.90[d]	+6.80	324
Polyglutamyl-BPA	Sodium cacodylate	6.3	−9.79	+7.28	290

[a] BPA = bovine plasma albumin. [b] RSA = rabbit serum albumin. [c] Mobility of the maximum gradient of the curve. [d] Mobility of the first moment of the curve. [e] Tris-(hydroxymethyl)-aminomethane hydrochloride.

290% increase indicating that a polyelectrolyte effect prevented complete ionization of the γ-carboxyl groups of the added polypeptide. The ionization of the β-carboxyl group of polyaspartic acid was spread over a comparatively large *p*H range[18] and a similar result is indicated for the polyglutamyl protein.

The experimental values for mobility change of proteins upon modification with polar amino acids did not correspond with the predicted values based on the empirical rule of mobility change of Longsworth and Jacobsen.[19] This rule is probably not valid for such a large change in the number of charged groups as was the case for these modified proteins. An increased frictional coefficient for the modified proteins would be expected because of increased hydration and attraction of secondary ion layers by the high ion density of the polypeptidyl protein surface. Because of the unknown nature of these effects no theoretical mobility change can be calculated.

Experimental

N-Carboxy-L-leucine and N-Carboxy-DL-phenylalanine Anhydrides.—These amino acid anhydrides were prepared in 95 to 99% yields according to the method of Farthing.[20]

Polymerization of N-Carboxy-L-leucine Anhydride.—One-gram portions of N-carboxy-L-leucine anhydride were polymerized in 100-ml. volumes of 0.07 *M* bicarbonate buffer, 0.07 *M* phosphate buffer (*p*H 7.4) and 0.07 *M* cacodylate buffer (*p*H 7.4), at 4° for 48 hours. The water-soluble portions of the polypeptide were used to determine the percentage hydrolysis by the ninhydrin method.

N-Carboxy-L-lysine Anhydride Hydrobromide.—11.5 g. of ε-carbobenzoxy-N-carboxy-L-lysine anhydride, prepared according to the method of Bergmann, *et al.*,[21] was dissolved in 100 ml. of purified dioxane. To remove the ε-carbobenzoxy group, hydrogen bromide was bubbled into the solution for 40 minutes at room temperature according to the method of Ben-Ishai and Berger.[13] The N-carboxy-L-lysine anhydride hydrobromide was precipitated as a sticky white solid by addition of 5 volumes of anhydrous ether. The solvent was decanted and the precipitate was washed with anhydrous ether. The product formed carbon dioxide

(18) A. Katchalsky, N. Shavit and H. Eisenberg, *J. Polymer Sci.*, **13**, 69 (1954).

(19) I. J. Longsworth and C. F. Jacobsen, *J. Phys. Colloid Chem.*, **53**, 126 (1949).

(20) A. C. Farthing, *J. Chem. Soc.*, 3213 (1950).

(21) M. Bergmann, L. Zervas and W. F. Ross, *J. Biol. Chem.*, **111**, 245 (1935).

upon the addition of water indicating that the anhydride ring was intact.

Polylysyl-bovine Plasma Albumin.—Two grams of crystalline bovine plasma albumin (Pentex, Inc., Kankakee, Ill., Lot A 1201) dissolved in 100 ml. of cold saturated sodium bicarbonate solution, was added to the freshly prepared N-carboxy-L-lysine anhydride hydrobomide, which dissolved in a few minutes. In other preparations the anhydride was divided into several portions and the protein solution added successively to each batch. The reaction mixture was allowed to stand 4 to 5 hours in the cold, dialyzed exhaustively against distilled water, followed by electrodialysis. The protein solution was then lyophilized and stored in the cold.

N-Carboxy-L-glutamic Acid Anhydride.—Ten grams of γ-benzyl-L-glutamic acid[22] was converted to γ-benzyl N-carboxy-L-glutamic acid anhydride in a 99% yield by the method of Farthing.[20] The anhydride (9.9 g.) was dissolved in 100 ml. of dioxane and the γ-benzyl group removed by hydrogenation at room temperature in the presence of 2 g. of 10% palladized charcoal. After 24 hours, when hydrogen uptake was complete and theoretical amount of hydrogen was consumed, the catalyst was filtered, washed with dioxane and the filtrate containing the anhydride was concentrated *in vacuo* to a colorless viscous oil.

Polyglutamyl-bovine Plasma Albumin.—Two grams of crystalline bovine plasma albumin dissolved in 100 ml. of cold saturated sodium bicarbonate solution was added to the freshly prepared N-carboxy-L-glutamic acid anhydride. After standing overnight in the cold, the reaction mixture was dialyzed exhaustively against distilled water and lyophilized.

Poly-L-leucyl- and Poly-DL-phenylalanyl-proteins.—Four hundred mg. each of finely powdered N-carboxy-L-leucine and N-carboxy-DL-phenylalanine anhydrides was suspended in 100 ml. of cold $1/_{15}$ *M* sodium bicarbonate buffer containing 2.0 g. of crystalline bovine plasma albumin and magnetically stirred for 48 hours. The reaction mixtures were centrifuged at 18,000 r.p.m. using No. 30 head of the Spinco model L centrifuge. The supernatant was dialyzed exhaustively against cold distilled water and lyophilized.

Preparation of Protein Solutions for Electrophoresis.—An approximately 1% (w./v.) solution of the lyophilized protein was dissolved in 0.1 ionic strength buffer and dialyzed with agitation against two changes of the same buffer. Samples of lyophilized protein dried over phosphorus pentoxide at 100° and 0.03 mm. pressure to constant weight showed an average loss of 6% moisture. The electrophoretic analyses were carried out on 0.94% (w./v.) solutions to correct for this 6% moisture.

Electrophoretic Analysis.—A Spinco model H electrophoresis-diffusion apparatus was employed for the electrophoresis measurements. All experiments were conducted in a 1.8-ml. capacity quartz micro Tiselius cell equipped with Alberty electrodes at a bath temperature of 2.07°. Conductivity measurements were made at 0° in a No. 038-0081 electrolytic conductivity cell (Perkin-Elmer Corp., Norwalk, Conn.) using a L. and N. portable conductivity bridge.

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(22) W. E. Hanby, S. G. Waley and J. Watson, *J. Chem. Soc.*, 3239 (1950).

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, UNIVERSITY OF OREGON]

The Ionization of Cysteine[1]

BY GEORGE GORIN

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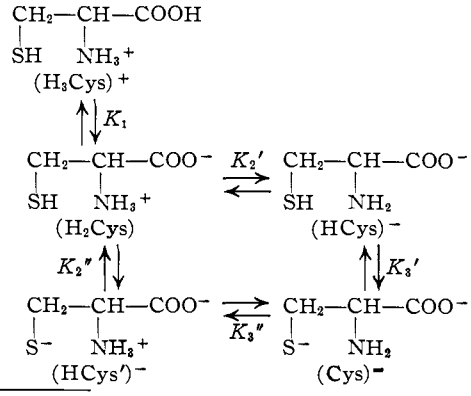
The ultraviolet absorption spectra of thioglycolic acid and its anion $^-SCH_2COO^-$ have been examined: a large increase in absorption toward longer wave lengths accompanies the ionization of the -SH group. Cysteine hydrochloride develops a similar absorption during the titration of its second proton (*pK* 8.27 at 25° in 0.10 *M* potassium chloride) and it is concluded that the ion $^-SCH_2CH(NH_3^+)COO^-$ is produced in this process. This ion may be in equilibrium with its tautomer, $HSCH_2CH(NH_2)COO^-$, but the presence of the latter can be estimated only on the basis of a rather uncertain assumption.

The mode of ionization of the amino acid cysteine has been the subject of considerable discussion, and the main point of uncertainty is of determining importance for defining the ionization of this substance in biological systems. Since cysteine, in its free and combined forms, plays an important role in the biochemistry of living organisms,[2] a clarification of the problem is very desirable.

The status of present knowledge may be summarized in the scheme



There is general agreement that the carboxyl hydrogen in $(H_3Cys)^+$ is the first to ionize, but there is a difference of opinion concerning the subsequent steps: (a) Cohn and Edsall,[3] in their classical work, indicate that the second proton comes from the ammonium group and the third from the thiol, giving, successively, $(HCys)^-$ and $(Cys)^=$; (b) Calvin[4] suggests the opposite view, that the ionization proceeds from (H_2Cys) through $(HCys')^-$ to $(Cys)^=$; and (c) Ryklan and Schmidt[5] propose that $(HCys)^-$ and $(HCys')^-$ are formed contemporaneously and in nearly equal quantities.

As was pointed out by Edsall,[6] the ionization constants which are determined experimentally from measurements of the hydrogen ion activity are related to those of the scheme above by the relations, $K_2 = K_2' + K_2''$; $1/K_3 = 1/K_3' + 1/K_3''$, $K_2K_3 = K_2'K_3' = K_2''K_3''$; the respective views are equivalent to making the assumptions, for (a), that $K_2' >> K_2''$, for (b), that $K_2'' >> K_2'$, and, for (c), that $K_2' \sim K_2''$. Recently, Grafius and

(1) Presented at the 128th National Meeting, American Chemical Society, Minneapolis, Minn., September, 1955.

(2) E. S. G. Barron, *Advances in Enzymology*, **11**, 201 (1951).

(3) E. J. Cohn and J. T. Edsall, "Proteins, Amino Acids and Peptides," Reinhold Publ. Corp., New York, N. Y., 1943, p. 85.

(4) M. Calvin, "Mercaptans and Disulfides," in "Glutathione," Academic Press, Inc., New York, N. Y., 1954, pp. 8–11.

(5) R. L. Ryklan and C. L. A. Schmidt, *Arch. Biochem.*, **5**, 89 (1944).

(6) J. T. Edsall, quoted in ref. 5.