acid (0.6 molar). The solution was swirled in an ice-bath and propionic anhydride (3.0 gram) was added from a dropping funnel during 20 minutes. After 2.5 hours at room temperature, the solution was decanted from a gummy placque and treated with 2 ml. of water during 2–3 minutes. After 90 minutes, isoamylamine (6.8 ml.) was added slowly. The solution was treated with a total of 350 ml. of anhydrous ether. After 18 hours in the ice-box a crude product (5.3 g., m.p. 145–147°) was collected on a filter. The recrystallized product melted at 157–158° with decomposition.

Anal. Calcd. for $C_6H_{11}NO_4$: C, 44.72; H, 6.88; N, 8.69. Found: C, 44.71; H, 6.90; N, 8.94.

***O*-Acetyl-DL-homoserine** was prepared from homoserine and acetic anhydride in perchloric acid by the same procedure[7] as described above in 75% yield, glistening plates, m.p. 183–185° dec.

Anal. Calcd. for $C_6H_{11}NO_4$: C, 44.72; H, 6.88; N, 8.69. Found: C, 44.50; H, 6.89; N, 8.48.

PITTSBURGH, PENNA.

[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF WISCONSIN]

*p*H Titration Studies of Polypeptidyl Proteins[1]

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*p*H titration curves of bovine and rabbit albumin which had been modified by the chemical attachment of polypeptides of glycine, leucine, phenylalanine, glutamic acid or lysine were determined by a micro continuous *p*H titration method. The number of polypeptide chains added and the average chain length were calculated. Unusual titration behavior of polylysyl bovine albumin was interpreted as an interaction of the polylysyl residues with tyrosine groups of the protein.

Changes in the number and types of ionic groups of a protein are best indicated by a study of its *p*H titration curve. Ionizations within certain ranges of *p*H may be assigned to certain amino acid residues[3]; from the number of hydrogen ions bound within a certain *p*H range, a quantitative estimate of the number of such groups may be made. This paper will report changes in the *p*H titration behavior of bovine and rabbit albumins which had been modified by the addition of extra amino acid residues linked as polypeptide chains to the original protein.

Stahmann and Becker[4] developed the preparation of water-soluble polypeptidyl proteins by reaction of N-carboxyamino acid anhydrides with proteins in buffered aqueous solution. This procedure yields proteins containing new amino acid residues attached through peptide bonds to the α- and ϵ-amino groups of the original protein.

On the basis of *p*H titration or treatment with 2,4-dinitrofluorobenzene, Becker and Stahmann[5] concluded that approximately one-third of the ϵ-amino groups of bovine albumin had reacted with N-carboxyglycine anhydride to form glycine polypeptides terminated with α-amino groups. The methods of Tsuyuki, *et al.*,[6] permitted the preparation of proteins with additional residues of the ionic amino acids glutamic acid and lysine. Greater changes in the *p*H titration behavior would be expected in these preparations as compared to modifications with neutral amino acids.

A careful study of the *p*H titration curve of bovine albumin was made by Tanford, Swanson and Shore.[7] We have used a rapid, albeit less accurate method of *p*H titration developed by R. M. Bock to determine the titration curve of bovine albumin and rabbit albumin as well as both albumins which had been modified by the addition of polypeptides of glycine, leucine, phenylalanine, glutamic acid or lysine.

Experimental

Preparation of Polypeptidyl Proteins.—Most of the polypeptidyl proteins were prepared by treating the protein with the N-carboxyamino acid anhydride in bicarbonate buffer at 4° as described by Tsuyuki, Van Kley and Stahmann.[6] The polyglycyl proteins were prepared by Dr. R. R. Becker[5] in phosphate buffer. Protein preparations were stored at −20° as a lyophilized powder. The increase in amino acid content was determined by microbiological assay of an HCl hydrolyzate.[8] Calculations for bovine albumin were based on a molecular weight of 69,000 and the amino acid analysis of Stein and Moore.[9] In the absence of physical measurements of the molecular weight of rabbit serum albumin, we have assumed its molecular weight to be 69,000. Calculations were based on the amino acid analysis of Schneiderman, Greene, Schieler, McClure and Dunn[10] except for the lysine values. These were obtained from the Wisconsin Alumni Research Foundation. This was done since the titration studies in the lysine range did not agree with the lysine content reported in the previously cited paper. The microbioassay value for lysine reported in our paper was performed on a sample obtained from the same source as the titration sample.

Apparatus used for Titration.—A titration apparatus requiring only 1 ml. of solution was used; this titrator was designed by Dr. R. M. Bock of the Biochemistry Department, University of Wisconsin. The sample is placed in a plastic centrifuge tube which is placed inside a turbine stirrer. A Leeds and Northrop all-purpose glass electrode fits inside the titration tube. Two polyethylene capillary tubes are taped onto the side of the electrode; one filled with

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(2) National Heart Institute Fellow, 1956–1957.

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(8) Several of the microbiological assays were performed by Drs. G. Lewis, F. Hepburn and J. Gupta in the laboratory of Prof. Elvehjem in the Biochemistry Department of the University of Wisconsin; the remainder of the assays were carried out by Dr. Maria Berger and Dr. B. V. Kline of the Wisconsin Alumni Research Foundation. We express our appreciation to these groups for the assays.

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saturated KCl acts as a salt-bridge to the calomel reference electrode, the other is connected to a modified Gilmont ultramicroburet equipped with a synchronous motor drive and filled with titrant. The turbine is rotated to provide stirring by means of thermostated water from a circulating pump; this water also maintains constant temperature. The *p*H is recorded on a Leeds and Northrup recording *p*H meter. Titrations were performed at 23°.

Preparation of Samples for Titration.—Eight to 17 mg. of lyophilized protein material was weighed directly into the titration tube and dissolved in 1 ml. of 0.15 *M* KCl solution; the observed *p*H was recorded as pH_0. Separate portions of the protein were dried to constant weight at 105° and corrections for moisture content were applied to the weight of sample taken for titration. The *p*H was raised to *p*H 11.2 to 12 by addition of a small amount of 1.5 *N* KOH. Standard HCl was added with the buret and the titration curve recorded until *p*H 2 was reached; no more than 0.08 ml. was used in any titration. A blank of 1 ml. of 0.15 *M* KCl was also run.

Calculation of Results.—After correction of the sample titration curve for hydrogen ion uptake by the solvent alone, regions of the curve were assigned to specific groups of the protein as follows[3]: *p*H 2–6, carboxyl, *p*H 6–8, imidazole + α-amino; *p*H 8–12, ε-amino + phenolic.

The microequivalents of acid bound in each of these regions was measured. The weight of protein used divided by the molecular weight of the protein (calculated for the modified proteins from the amount of amino acid added) gave the moles of protein. The microequivalents of acid divided by the micromoles of protein gave the number of each group titrated per mole of protein. The number of α-amino groups was calculated by subtracting the number of imidazole groups reported for the unmodified proteins.[7,10] ε-Amino groups were calculated by subtracting the number of phenolic groups.[7,10] The curves were also corrected for overlapping of titration regions when a large number of groups was observed.

Results and Discussion

Figure 1 shows the titration curve obtained with 16.8 mg. of unmodified bovine albumin; the shape of the corrected curve is very similar to the curve of Tanford[7] at an ionic strength of 0.15 calculated from at least 80 separate measurements. Calculation of the number of titratable groups present in two typical titrations is given in Table I. The agreement between samples and with the results of Tanford[7] may be considered quite reasonable in view of the method and small samples which were used.

TABLE I

NUMBER OF IONIC AMINO ACID RESIDUES PER MOLE OF BOVINE AND RABBIT ALBUMIN

Method	Carboxyl[a]	Imidazol[b]	α-Amino[c]	ε-Amino[d]
	Bovine albumin			
Titration	118	15	1	59
Titration[7]	106	17	1	60
Chromatography[9]	104	18	..	60
	Rabbit albumin			
Titration	89	13	1	55
Microbiological assay[8,10]	87	13	..	45

[a] *p*H 2 to 6. [b] *p*H 6.0 to 7.5. [c] *p*H 7.5 to 8.0. [d] *p*H 8.0 to 12.0 corrected for 20 tyrosine groups.

Table II summarizes the data obtained by titration of various polypeptidyl bovine albumins. The number given after the polypeptide indicates the moles of amino acid added per mole of protein as determined by microbiological assay. Table III summarizes the data from the titration of the various polypeptidyl rabbit albumins. A comparison of the titration studies to the microbioassays reveals good agreement for the unmodified rabbit

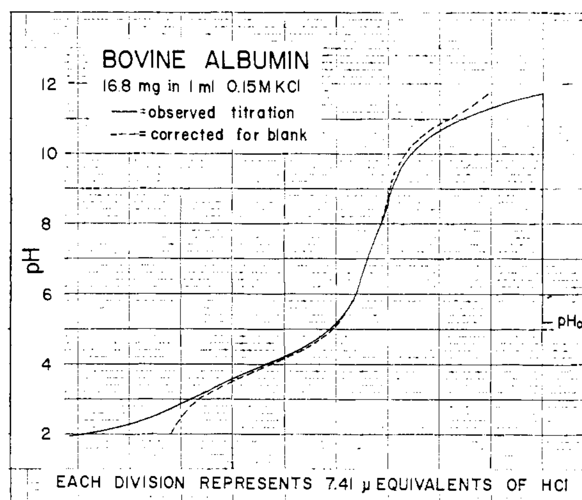

Fig. 1.—Continuous titration curve of bovine albumin.

albumin. In all cases an increase in the number of α-amino groups was found and a corresponding decrease in ε-amino groups indicating that some of the ε-amino groups were acylated with the anhydride to form α-amino groups at the end of a polypeptide chain. Table II also contains calculations of the number of polypeptide chains added based on the number of additional α-amino groups and calculations of the average chain length based on the increase in amino acid content and the number of chains added.

Titration studies with polyglycyl and polyleucyl bovine albumins were limited by the low solubility of the preparations in 0.15 *M* KCl necessitating small samples. Larger amounts of glycine than of leucine were added during modification but the additional amounts were present as longer polypeptide chains rather than as more chains. Some variation in the number of ε-amino groups reacted may be noted.

Preparations of polyphenylalanyl bovine and rabbit albumins were quite similar to other preparations with non-ionic amino acids except when DL-phenylalanine was used for modification. In the case of bovine albumin approximately 20 new polypeptide chains were added which was the largest number observed in any bovine albumin preparation. Figure 2 illustrates the titration curve for this preparation; the changes in the region of *p*H 6 to 12 are quite apparent. An increased number of α-amino groups explains the greater change in electrophoretic mobility previously reported for this preparation.[11] Polyglutamyl C1 rabbit albumin had 24 new polypeptide chains added and polyphenylalanyl C1 rabbit albumin had 19 new polypeptide chains added. Apart from these two protein modifications the modified rabbit albumins were modified to the same extent as the modified bovine albumins.

The polyglutamyl proteins showed a greatly increased buffering in the acidic region as would be expected by the addition of glutamic acid residues. When a large number of carboxyl groups was added, the titration extended beyond the *p*H region

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TABLE II

NUMBER OF IONIC GROUPS AND ADDED POLYPEPTIDE CHAINS IN POLYPEPTIDYL BOVINE ALBUMINS

Protein, residues amino acid added per mole and preparation no.	No. of groups titrated per mole protein Carboxyl[a]	α-Amino[b]	ε-Amino[c]	No. of new polypeptide chains[d]	Av. no. of residues per chain[e]
Bovine Albumin	118	1	59	0	0
Polyglycyl$_{232}$ Bovine Albumin A3	129	13	63	12	19
Polyglycyl$_{261}$ Bovine Albumin A4	112	19	38	18	15
Poly-DL-leucyl$_{46}$ Bovine Albumin F1	113	11	45	10	5
Poly-L-leucyl$_{49}$ Bovine Albumin C2	121	18	51	17	3
Poly-L-phenylalanyl$_{31}$ Bovine Albumin F2	107	7	51	6	5
Poly-L-phenylalanyl$_{36}$ Bovine Albumin F1	117	10	44	9	4
Poly-DL-phenylalanyl$_{48}$ Bovine Albumin C2	116	22	32	21	2
Poly-L-glutamyl$_{13}$ Bovine Albumin F1	133	3	53	2	7
Poly-L-glutamyl$_{41}$ Bovine Albumin C6	164	7	66	6	7
Poly-L-glutamyl$_{73}$ Bovine Albumin C4	199	12	52	11	7
Poly-L-glutamyl$_{218}$ Bovine Albumin C5	317	11	64	10	22
Poly-L-glutamyl$_{275}$ Bovine Albumin C2	377	10	48	9	30
Poly-L-lysyl$_{2}$ Bovine Albumin C3	122	f			
Poly-L-lysyl$_{14}$ Bovine Albumin C1	118	f			

[a] pH 2 to 6; corrected for groups titrating above pH 6 when many are added to the protein. [b] pH 6 to 8; corrected for imidazole groups.[7] [c] pH 8 to 12; corrected for 19 tyrosine groups.[7] [d] Increase in α-amino groups over assumed one[7] in unmodified protein. [e] Number of residues added divided by increase in α-amino groups. [f] Titration of imidazole and amino groups apparently shifted to higher pH regions.

usually assigned to carboxyls. The ionization in aqueous solution of the β-carboxyl groups of polyaspartic acid takes place over a large pH region as a result of the polyelectrolyte character of the polymer,[12] a similar polyelectrolyte effect may be operative here. A comparison of the titration method with microbiological assay for increase in glutamic acid content is shown in Table IV; the results are considered to agree within the experimental error involved in both methods.

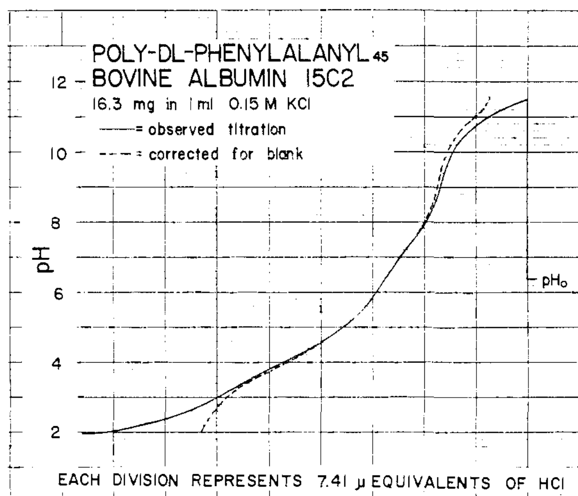

Fig. 2.—Continuous titration curve of poly-DL-phenylalanyl[45] bovine albumin C_2.

The number of additional ionized carboxyl groups present at a certain pH was calculated from the titration curve and correlated with changes in the electrophoretic mobility; these results are discussed in the accompanying paper.[13]

The titration region from pH 6 to 12 was greatly changed in polylysyl bovine albumin preparations; a typical curve is shown in Fig. 3. Despite the addition of new α-amino groups, uptake of hydrogen ion in the region usually associated with imidazole and α-amino groups was greatly diminished and apparently shifted to a higher pH region. In the accompanying paper[13] an increase in ultraviolet absorptivity in polylysyl bovine albumin preparations is discussed; consideration of this phenomenon with the titration results indicates a possible interaction between tyrosine residues which are mainly responsible for the characteristic 278 mμ absorption of proteins and the added polylysine residues.

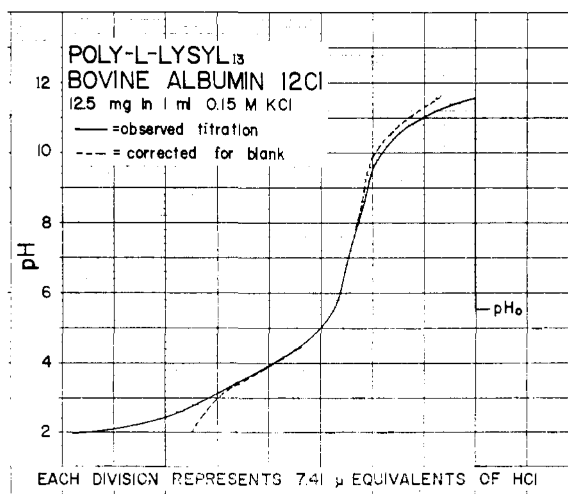

Fig. 3.—Continuous titration curve of poly-L-lysyl$_{13}$ bovine albumin C.

A wide distribution of chain lengths was found for the product of amine-initiated polymerization of γ-benzylglutamic acid anhydride in dioxane solution.[14] Polymers prepared in dimethylformamide showed much less polydispersity and ap-

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TABLE III

NUMBER OF IONIC GROUPS AND ADDED POLYPEPTIDE CHAINS IN POLYPEPTIDYL RABBIT ALBUMIN

Protein, residues amino acid added per mole and preparation no.	No. of groups titrated per mole protein			No. of new polypeptide chains[d]	Av. no. of residues per chain[e]
	Carboxyl[a]	α-Amino[b]	ε-Amino[c]		
Rabbit Albumin	90	3	55		
Poly-L-glutamyl$_{[illegible]}$ Rabbit Albumin C1	214	27	55	24	5
Poly-L-glutamyl$_{47}$ Rabbit Albumin C3	143	14	47	11	4
Poly-DL-phenylalanyl$_{92}$ Rabbit Albumin C1	121	22	51	19	5
Poly-DL-phenylalanyl$_{121}$ Rabbit Albumin C3	107	17	31	14	9
Poly-L-leucyl$_{52}$ Rabbit Albumin C3	85	12	24	9	6
Poly-L-leucyl$_{48}$ Rabbit Albumin C4	101	16	40	13	4
Poly-L-leucyl$_{29}$ Rabbit Albumin C5	87	13	26	10	3
Poly-L-lysyl$_{12}$ Rabbit Albumin C2	108	9	71	..	..

[a] *p*H 2 to 6; corrected for groups titrating above *p*H 6 when many are added to the protein. [b] *p*H 6 to 8; corrected for imidazole groups.[7] [c] *p*H 8 to 12; corrected for 19 tyrosine groups.[7] [d] Increase in α-amino groups over assumed one[7] in unmodified protein. [e] Number of residues added divided by increase in α-amino groups.

proached the expected Poisson distribution of chain lengths.[15] There is no information on the polydispersity of chain lengths in polypeptides prepared in buffered aqueous solution; no attempts were made to ascertain the distribution of chain lengths in the various polypeptides attached to one protein molecule. Only an average chain length can be calculated from the presently available data on the number of polypeptide chains and the number of moles of amino acid added per mole of protein. Also no information is obtained on the distribution of the polypeptide chains on the protein molecule. A maximum of 21 of the 60 ε-amino groups in bovine albumin and 24 of the 55 ε-amino groups in rabbit albumin reacted with the N-carboxyamino acid anhydride. It may be proposed that steric hindrance of the protein surface by the first molecules of anhydride to react may prevent other nearby ε-amino groups from reacting, thus providing a random distribution of polypeptide chains on the protein molecule.

TABLE IV

INCREASE IN GLUTAMIC ACID; MOLES PER MOLE PROTEIN

Preparation no.	Microbiological assay	*p*H Titration
F1	13	15
C6	41	46
C4	73	81
C5	218	199
C2	275	257

The *p*H titration data for these preparations show that there was not a significant amount of the termination reaction which has been shown to occur during the polymerization of N-carboxyamino acid anhydrides in organic solvents.[16] This side-reaction produces a terminal carboxyl rather than the expected α-amino amino group which would result in an increase of the total number of carboxyl groups and a decrease of amino groups even though neutral amino acid residues were added to the protein. If such a reaction did occur, the amount was less than the experimental error in the titrations.

The method of continuous titration with small samples has not been used previously with proteins. It does not provide the accuracy of measurements on many individual samples but the complete titration curve of a protein may be obtained in approximately 30 minutes on 10 to 15 mg. of material. The method is proposed as being useful for studies when large changes in the ionizable groups are expected or if only a limited amount of protein material is available.

Acknowledgment.—Several of the samples used in this study were made available through the cooperation of Dr. H. Tsuyuki.

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