CHEMICALLY MODIFIED MYOGLOBIN:

PREPARATION OF SPERM WHALE AND HORSE ACETYLATED MYOGLOBINS.

STUDIES ON TITRATION CURVES

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ABSTRACT

Sperm whale and horse myoglobins were acetylated using acetic anhydride. The degree of modification of the ϵ-amino groups of lysine residues was determined by treating the acetylated myoglobin with dinitrofluorobenzene to block the unmodified lysine residues followed by amino acid analysis. Some physical studies were made on the acetyl and succinyl derivatives of sperm whale and horse myoglobins. (1) The acetyl derivatives of sperm whale and horse myoglobins were found to be more negative than the native myoglobins, when passed through a cation exchange column. (2) The following isoionic points were found: sperm whale (n) Mb, 8.07 $\pm$ 0.01; sperm whale Ac-Mb, 6.36 $\pm$ 0.06; sperm whale Suc-Mb, 5.96 $\pm$ 0.06; horse (n) Mb 7.44 $\pm$ 0.01; horse Ac-Mb, 5.47 $\pm$ 0.01. (3) The basic titration curves of the acetyl and succinyl derivatives did not show the titration of lysine residues as in the case of native myoglobins. The titration curves of the myoglobin derivatives were consistent with the unfolding of the protein molecule upon acetylation or succinylation.

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LIST OF SYMBOLS AND ABBREVIATIONS

(n) Mb: Native Myoglobin

Ac-Mb: Acetylated Myoglobin

Suc-Mb: Succinylated Myoglobin

Gn-Mb: Guanidinated Myoglobin

Mb IV: Myoglobin component IV (Edmundson & Hirs, 1962)

Mb III: Myoglobin component III (Edmundson & Hirs, 1962)

GDMP: 1-guanyl-3,5-dimethyl pyrazole nitrate

DNFB: Dinitrofluorobenzene

DNP: Dinitrophenyl

pI: Isoionic point

x'; μequivalents of acid or base added

x: Corrected x' for the values of water

ν_{H^+} : Equivalent of acid or base added / mole of myoglobin

a-x: μequivalents of acid or base added in the back titration

y: Corrected a-x for the values of water

INTRODUCTION

Myoglobin is the protein found in the muscles of all vertebrates giving the muscle the characteristic red color. Most of the X-ray studies until recently have been done on sperm whale myoglobin, this is because whale meat is one of its richest sources. (Perutz, 1962).

Myoglobin is made of two moities, globin and heme. The molecular weight is about 18,000. Globin is the protein part and consists of 153 amino acids. The heme part is made of tetrapyrrolic ring and an atom of iron which is coordinated to the four pyrrolic nitrogens. The fifth coordination site of iron is occupied by the nitrogen of the imidazole of the histidine residue in the globin. The sixth coordination site is occupied by a molecular oxygen or a water molecule. (Perutz, 1962). Different species of myoglobin have slightly different composition of amino acids (Table 1).

Of the 153 amino acids of sperm whale myoglobin 118 residues are folded in eight segments of right handed α-helices having the letters A - H. The helix is 7 - 24 residues long. The helical segments are joined by two sharp corners with no helical residues such as proline and by five non-helical segments 1 - 8 residues long. There is also a non-helical tail at the end of the carboxyl end of the chain containing five residues. The whole structure is roughly a compact triangular prism with dimensions about 45 x 35 x 25 $\mathring{A}$. There are no channels in the molecule and the internal space is small. Almost all residues having polar groups in the side chain such as Lys, Glu etc.

Table I

Amino Acid Composition of Sperm Whale and Horse Myoglobins

Amino Acid	Number of residues in sperm whale Mb*	Number of residues in horse Mb+
Asp	6	11
Asn	2	
Glu	14	19
Gln	5	
Ser	6	5
Thr	5	7
Met	2	2
Pro	4	4
Gly	11	16
Ala	17	16
Leu	18	18
Ileu	9	9
Phe	6	7
Tyr	3	2
Trp	2	2
His	12	11
Lys	19	19
Arg	4	2
NH_3	(7)	(7)

* Edmundson (1965)

+ Theorell & Akeson (1960)

are on the surface of the molecule except those having specific function in the interior of the molecule such as the histidine residue which is linked to the heme. The non-polar groups such as Val, Leu, Phe, etc. constitute the interior of the molecule. They are very compact and in Van der Waals contact with each other. The heme is almost disposed normally to the surface of the molecule. The side of the heme group which contains the polar propionic acid groups, is at the surface and the rest of the heme is buried deeply within the myoglobin molecule (Kendrew, 1963).

Chemical modification is a tool to study the groups which give the protein its biological activity. Modification can also be used to elucidate some details about the structure of the protein especially the role of some critical groups which give the protein its stability. In 1935 Greenstein prepared the amide and the ϵ-Guanido α-amino carponyl glutamic acid derivatives of the dipeptide lysyl-glutamic acid. Fraenkel-Conrat *et al.* (1949) reported that acetylation of most of the amino groups did not inactivate trypsin and its inhibitor ovomucoid; but acetylated trypsin was no longer susceptible to inhibition by ovomucoid or the ovomucoid acetyl derivative. It was found that esterification of carboxyl and phenolic groups diminished the inhibiting action of ovamucoid from which they concluded that carboxyl and phenolic groups were essential for the inhibiting action of ovamucoid.

Chervenka & Wilcox (1956a) found that the free imino groups of proline and hydroxy proline; the sulfhydryl, α-amino, and ϵ-amino groups of amino acids in chymotrypsinogen reacted quantitatively with CS_2 under mild conditions of temperature and pH. The kinetic analysis of the

reaction of CS_2 with chymotrypsinogen revealed that the α-amino group of the N-terminal half cysteine residue was much more reactive than the other groups. The same authors (1956b) prepared the guanidinated derivative of chymotrypsinogen using O-methylisourea as the guanidinating reagent. They found that O-methylisourea was a specific reagent for ϵ-amino groups. Guanidinated chymotrypsinogen was found to be less stable in aqueous solution, had more negative electrophoretic mobility, and a higher sedimentation constant than the native chymotrypsinogen.

Habeeb et al. (1958) studied the molecular structure effects produced in proteins after reaction with succinic anhydride. The characteristics of the succinylated protein, such as large negative electrophoretic mobility and increased intrinsic viscosity, indicated unfolding or expansion of the protein which was attributed to electrostatic repulsion produced by a large number of negative charges placed on the molecule.

In 1959 Habeeb et al. did studies on acetylated and guanidinated derivatives of some antibodies. Acetic anhydride was used for acetylation and O-methylisourea for guanidination. Acetylation of 40% of the ϵ - aminogroups of lysine residues of the antibodies led to complete loss of the antibody activity. The loss was attributed to the intramolecular electrostatic repulsion which caused group configurational changes in the protein rather than the blocking of the critical groups in the active sites of antibody molecule. This was confirmed by blocking the ϵ -amino groups with a guanido group which has no effect on the charge of the protein. The guanidinated

derivative showed no significant loss in the activity of the antibody.

Habeeb (1959) introduced a new reagent (1-guanyl-3,5-dimethyl pyrazole nitrate) for guanidination of proteins. He studied the course of guanidination of some proteins and the properties of the guanidinated derivatives (1960). The reagent 1-guanyl-3,5-dimethyl pyrazole nitrate was found to react specifically with the ε -amino group of the lysine residue without affecting other amino acids (Habeeb, 1961).

Singer (1959) prepared antibody conjugates using m-xylylene diisocyanate as coupling agent. Schick & Singer (1961) compared the types of covalent linkage between two proteins by using different coupling agents. They found that conjugates prepared with m-xylylene diisocyanate as coupling agent were partly covalent while with toluene-2,4-diisocyanate as coupling agent, conjugates were exclusively covalent. Luisada-Opper et al. (1961) prepared azoproteins by coupling bovine serum albumin and its acetylated derivative with arsanilic acid and p-aminobenzoic acid. The coupling with arsanilic acid was inversely proportional to the percentage of O-acetylation while coupling with diazotized p-amino benzoic acid was found to be independent of the percentage of O-acetylation. A comparison of azo N-acetyl bovine serum albumin and azo native bovine serum albumin permitted the evaluation of coupling of ε -amino groups of lysine residues since they were blocked upon acetylation.

In 1960 Abadi & Wilcox studied the reaction of α -chymotrypsinogen with N-acetyl DL-Homocysteine thiolactone. They measured the number of moles of homocysteine incorporated into the protein and found

that at pH 7.2 and 25°, the incorporation was very small, at pH 7.5 and 25° the incorporation was 2 moles per mole of protein and at pH 8.0 it was 5.6 per mole. Electrophoresis showed gradual acylation of the protein. Doscher & Wilcox (1961) prepared diazoacetamide in 8 - 14% yield and used it in esterification of α-chymotrypsinogen and simple carboxylic acids. The rate of reaction of chymotrypsinogen with the first one or two molecules of the reagent was significantly higher than the rate with the succeeding molecules. Electrophoresis and chromatography showed that the preparationswere mixtures of at least two components. Amino acid analysis failed to show the specificity of the reaction. UV spectra showed that the tyrosine residues were not modified.

Wofsy & Singer (1963) studied the effect of amidination of the ε-amino groups of the lysine residues on the antibody activity and on the physical properties of some proteins. Exhaustive amidination produced few remarkable effects on the biological activity of the antibodies studied. Lysine residues were not critical sites in any of the antibodies studied. Practically no sign of structural alteration in the proteins appeared up to 90% amidination and only small effects were observed with exhaustive amidination, such as increased levorotary behaviour and a 7 - 9% increase in the UV extinction coefficient. These effects were attributed by Habeeb (1966) to conformational changes in the protein due to reorganization of non-covalent bonds that stabilize the protein molecule.

Banaszak *et al.* (1963) prepared guanidinated sperm whale myoglobin using O-methylisourea as guanidinating agent. The hydrodynamic

properties, titrations and reactivity towards p-nitrophenyl acetate and bromoacetate of the guanidinated myoglobin were compared with that of the native.

Banaszak & Gurd (1964) prepared the carboxymethyl derivative of sperm whale metmyoglobin and studied the reactivity of the adjacent histidine residues (EF_4 & EF_5). The EF_4 was found to react with the modifying agent while the EF_5 was not which suggested that the EF_4 histidine residue is exposed to solution while EF_5 is not.

Reihm & Scheraga (1966) studied the effect of blocking the ϵ-amino group of lysine residues with acrylonitrile on the structure and activity of ribonuclease. The derivative was inactive to substrate but it possesed similar physiochemical properties to that of the native protein.

In 1966 Hoare & Koshland introduced a procedure for selective modification of carboxyl groups in proteins. The procedure which involved soluble carbodiimide and a modifying agent, glycine methyl ester, lead to rapid and quatitative modification of carboxyl groups under mild conditions.

Atassi (1966) studied the role of the amino groups and the C-terminal group in sperm whale myoglobin in the antigen-antibody reactions. After blocking the lysine residues with acetyl groups, the acetylated myoglobin failed to react with anti-myoglobin. This might be due to structural changes upon modification or due to blockage of the active sites. Contrary to Edmundson (1965) Atassi suggested that the C-terminal of sperm whale myoglobin is glutamine rather than glycine and it does not play a role in the antigen-antibody reaction.

He also reported the absence of any spectral changes in the acetylated myoglobin.

Habeeb (1966) studied the conformational changes upon modification of bovine serum albumin and human γ-globulin. The method of study was based on the assumption that conformational changes were in the vicinity of the disulfide groups. This will make the disulfide groups accessible for reaction with β-mercaptoethanol, sodium sulfite, and peraacetic acid. He suggested that conformational reorganization in the amidinated and guanidinated derivatives involved the reorganization of the non-covalent bonds that stabilize the protein molecule; while in the nitro-guanyl derivatives the reorganization might involve hydrophobic non-covalent bonds which were capable of counteracting the disrupting effect of electrostatic repulsion. In the case of the succinylated and acetylated proteins it was suggested that reorganization might be predominantly electrostatic in nature.

Atassi (1967) studied the reaction of myoglobin with 3,3-tetra-methylene glutaric anhydride. This reagent is specific for the modification of ϵ-amino groups of lysine residues. Electrophoresis showed that the derivative was homogenous and very negatively charged. The derivative had a different spectrum than the native myoglobin.

Proteins contain a variety of acidic and basic groups with different pK's. The number of titratable groups in most proteins with molecular weight less than 100,000 ranges from 20 - 250 groups. The titratable groups include the free carboxyl groups of glutamic and aspartic acids, the ϵ-amino group of lysine, the phenolic group of tyrosin, the imidazole of histidine, the guanidine of arginine, and

finally the N- and C-terminal residues. The hemoproteins contain titratable groups attached to the heme and a water molecule attached to the iron atom of the heme. Titrations are carried out in the pH range of 2 - 12. Outside this range most proteins are degraded. At any pH during the titration, there will be a number of titratable groups in their charged form (NH_3^+ or COO^-). Due to these electrostatic effects the titration of one group will be influenced by the other charged groups. As the pH changes during a titration, many groups will be titrated together. Due to the electrostatic interactions of the titratable groups the pK's of the groups will be lower at low degree of dissociation than at high degree of dissociation. The pK of any group will change during the course of titration and the resulting titration curve will be broader than that of a monobasic acid (Tanford, 1962).

There are many factors that influence the course of titrations in proteins other than the electrostatic effects. Titration curves of proteins depend on ionic strength and on the protein concentration, if there is a possibility of association. The reference point of the titration can be the isoionic point or the points of maximum proton charge and of minimum proton charge (Tanford, 1962).

Titration curves can be followed by pH measurements during the course of titration. Spectroscopic titrations are feasible for tyrosine residues and they can be used in following the dissociation of hydrogen ion in the $Fe(H_2O)^+$ group in hemo-proteins. However, it is extremely difficult to detect small changes in the absorbance of the imidazole group due to change in ionization, since the aromatic

residues (tyrosine, phenylalanine, tryptophan) absorb strongly in the UV, making it essentially impossible for the detection of such changes (Tanford, 1962).

Theorell & Akeson (1941) determined the titration curves of cytochrome c from beef heart and horse heart. The major problem in the titration was the three imidazole groups in the protein. They suggested that only one group of the three imidazoles was titrated on rather weak evidence. However, Paléus (1954) proved with more accurate data the suggestion of Theorell & Akeson. This lead to the conclusion that two of the three imidazoles are attached to the heme, consistent with the suggestion of Margoliash et al. (1959).

Wilcox (1961) studied the titration curves of α-chymotrypsin. He also obtained a partial titration curve for the guanidinated derivative. The main difference between the two was the disappearance of the ten-side chain amino groups. In 1962 Harstein & Hess studied the titration of the phenolic groups of α-chymotrypsin and its diisopropyl phospho derivative. All four tyrosyl groups were titrated in solvents that denature the protein.

There are three outstanding features in the titration of hemoglobin. First, four groups with pK near 8.0 were found to be titrated in ferrihemoglobin but not in ferrohemoglobin (German & Wyman Jr., 1937; Wyman, Jr. & Ingalls, 1941; George & Hanania, 1953). These groups represent the proton dissociation from the heme-iron atom, $Fe(H_2O)^+ \rightleftharpoons Fe(OH) + H^+$. The second feature is that the acidic region of the titration curve is time dependent and irreversible (Steinhardt & Zaiser, 1951), indicating an unfolding of the molecule, hence a decrease

in the electrostatic interactions. The third feature is that four groups which have a pK near 7.9 in hemoglobin titrate with a lower pK (6.7) in the hemoglobin-oxygen complex. These are the heme-linked imidazole groups (Tanford, 1962).

The titration curves of myoglobin have been given some special interest due to the complete knowledge of the structure of this protein. Breslow & Gurd (1962) presented a titration curve for sperm whale myoglobin. They used a method based on the catalysis of the hydrolysis of p-nitrophenyl acetate by uncharged imidazole groups to follow the dissociation of hydrogen ion from the imidazole group of the histidine side chains. They observed a sharp break in the titration curve at pH 4.5 with a marked uptake of hydrogen ion down to pH 4.0. The same sharp break was observed also by Steinhardt & Zeiser (1951) between pH 3 and 4 in horse ferrihemoglobin. The break was attributed by Breslow & Gurd to the titration of 6 imidazole groups of the histidine residues simultaneously. The titration curve exhibited a time dependent acid denaturation. Only six groups of the histidine residueswere titrated in native protein while the all 12 histidines were titrated in the denatured form, leading to the conclusion that 6 of the 12 imidazole groups are burried in the interior of the molecule in their uncharged form. Other groups in myoglobin have been studied in detail. The $Fe(OH_2)^+$ group dissociation was studied and its pK was determined by Theorell & Ehrenberg (1951), George & Hanania (1952), Breslow & Gurd (1962) from spectral changes which accompany the dissociation.

The ionization curves of tyrosine residues in sperm whale and horse myoglobin; horse and human hemoglobin (Hermans, 1962) were

determined by using difference spectra at 245 mu as a measure of the extent of ionization of the tyrosine residues. In myoglobin two of the three tyrosines could be titrated below pH 12.

The purpose of the present work was to modify myoglobin chemically and to compare the physical properties of the derivatives with that of the native. Acetylated and guanidinated derivatives of both sperm whale and horse myoglobin were prepared. Acetic anhydride was used as the acetylating agent and GDMP (1-guanyl-3,5-dimethyl pyrazole nitrate) as the guanidinating agent. These two modifications were chosen in order to study the effect of charge on reactivity and conformation of the myoglobin molecule. Acetylation blocks the ϵ-amino groups of lysine residues, converting their positive charge to zero charge. Guanidination blocksthe ϵ-amino groups of lysine residues without changing the formal charge. Along with these modifications succinylated sperm whale myoglobin was prepared and characterized by M. Beudjekian (Thesis, 1968). Succinylation blocks the ϵ-amino groups of lysine residues converting their positive charge to a negative one.

The acetyl derivatives of sperm whale and horse myoglobin were prepared. The attempts to get the guanidinated derivative failed. The degree of modification of the derivatives was determined by treating the modified myoglobins with DNFB (dinitrofluorobenzene) to block the unmodified lysine residues followed by amino acid analysis using the Technicon Auto-Analyzer.

For the purpose of physical characterization, titration curves of native and modified myoglobins were determined. The isoionic points were obtained by measuring the pH of the sample of myoglobin emerging

from the Dintzis column or the Sephadex G-25 column. CM-Sephadex C-50 chromatography was done to show the difference in the elution volumes of the native and the modified myoglobin, indicating differences in the charge on the molecule.

EXPERIMENTAL

Materials

Myoglobins. Sperm whale (skeletal muscle) and Equine ferrimyoglobins, Salt-free, lyophilized were purchased from Seravac Laboratory (Maidenhead, Berks, England) and used without further purification.

Succinylated sperm whale myoglobin. Sperm whale Suc-Mb was prepared by M. Beudjekian (Thesis, 1968).

Ninhydrin was purchased from Pierce Chemical Company (Rocford, Illinois).

Hydrindantin was prepared according to the procedure of Moore & Stein (1954b). To 20 g of ninhydrin in 500 ml of water at 90°, a solution of 20 g of ascorbic acid in 100 ml of water at 40° was added with stirring. Crystallization of hydrindantin started immediately and was allowed to continue for 30 minutes. During the next hour the solution was cooled under running tap water. The hydrindantin was filtered off, washed with water and dried to constant weight over P_2O_5 in a vacuum desiccator protected from light. The yield was 95%.

Ethyl cellusolve (oxitol) was purchased from Shell Oil Company, Beirut, and was used without further purification. The oxitol was found to be peroxide-free by testing with fresh 4% KI solution.

Dinitrofluorobenzene (BDH, Poole, England) was kept in a dark bottle in the cold room.

Polyoxyethylene (23) Lauryl ether (Brij 35 SP). (Atlas Chemical Industries, Wilmington, Delaware). The solution of Brij was made by

dissolving 100 g of Brij in 200 ml of water with warming.

Thiodiglycol was purchased from BDH, Poole, England.

Helium gas, dry, high purity, was purchased from Sherwood Overseas Corp. New York.

L-Amino acids were purchased from BDH, Poole, England.

CM-Sephadex C-50 (Pharmacia, Uppsala, Sweden). As quoted by the manufacturers, the capacity was 4.5 $\pm$ 0.5 meq/g and the particle size 40 - 120 μ.

Sephadex G-25 (Pharmacia, Uppsala, Sweden). As quoted by the manufacturers, the water regain is 2.4 g of water per g of dry gel.

Cation exchange resin, AG50W x 8, 20 - 50 mesh, purchased as the Na^+ cycle from BIO.RAD laboratories, Richmond, California. As quoted by the manufacturers the capacity is 5.1 meq/dry g and 1.7 meq/ml resin bed.

Anion exchange resin, AG1 x 8, 20 - 50 mesh, purchased as the Cl^- cycle from BIO.RAD Laboratories, Richmond, California. As quoted by the manufacturers the capacity is 3.2 meq/dry g and 1.4 meq/ml resin bed.

All other chemicals were of reagent grade. Distilled deionized water (specific conductance better than 10^{-6} ohm^{-1} cm^{-1}) was used throughout this work.

Methods

Chemical modification of myoglobins

Acetylation procedure

Sperm whale Mb and Horse Mb were acetylated according to the procedure of Habeeb et al. (1958). To a solution of 3 g of myoglobin

in 60 ml of water, 3 ml of acetic anhydride were added gradually with magnetic stirring at 0°. The pH was maintained around 8.0 by the addition of 1N NaOH using a small syringe. After about half an hour reaction time, the mixture was exhaustively dialyzed against water at 4° and lyophilized. The salt-free, dried modified myoglobin was stored in a tightly closed bottle in the cold room at 4°. The yields were: acetylated sperm whale Mb 2.17 g (87%); acetylated horse Mb 2.18 g (77%).

Guanidination procedure

The guanidinating reagent, 1-guanyl-3,5-dimethylpyrazole nitrate, was prepared according to the procedure of Bannard et al. (1958). To a refluxing solution of 2,4-pentanedione (12.5 g, 0.125 mole) in 50% aqueous ethanol (35 ml), aminoguanidine nitrate (17 g, 0.125 mole) was added over a period of half an hour and the resulting solution was heated under reflux for 2½ hours. The solution was allowed to stand overnight. The crystals which separated were collected, washed with ether and dried in vacuum. The product was crystallized from ethanol. The yield was 64%, m.p. 168°.

Guanidination of sperm whale and horse myoglobin was carried out according to the procedure of Habeeb (1961). GDMP (5 g) was dissolved in a few ml of water and the pH was adjusted to 9.5 with 1N NaOH. Mb solution was added to the reagent. The total concentration of the reagent was 0.25 M and that of Mb 3%. The reaction was carried out for 7 days at 0°. At the end of the second day a precipitate formed. At the end of the reaction time (7 days), the supernatant and the precipitate of the reaction mixture were dialyzed separately, exhaustively,

against water at 4° and lyophilized. The yields were: Guanidinated sperm whale Mb (supernatant) 2.17 g (72%), guanidinated sperm whale Mb (precipitate) 0.33 g (11%), guanidinated horse Mb (supernatant) 1.41 g (47%), guanidinated horse Mb (precipitate) 0.15 g (5%). Lyophilizations were done at 0.005 mm Hg using a Duo Seal vacuum pump model 1405 (The Welch Scientific Company).

CM-Sephadex C-50 chromatography

A glass chromatographic column, 25 cm x 1.27 cm diam, was packed with CM-Sephadex C-50 using 0.05 M phosphate buffer 6.85 which was also used as eluant. The sample for analysis was prepared in the same buffer and was made dense by adding a few crystals of sucrose. The sample (0.5 ml) was applied to the column with an Agla micrometer syringe (Burroughs Wellcome & Co., London, England) fitted with a thin plastic tubing. The outlet of the column was closed at the time of applying the sample. After application, the inlet cap of the column was tightly closed and elution was started by opening the column outlet.

A time-operated fraction collector (Gilson Medical Electronics, Middleton, Wisconsin) was used to cut the effluent into fractions. The volume of each fraction was obtained from the weight difference between the full and empty test tube. The effluent fractions were analyzed by reading absorbancy at 410 and 280 mu on a Zeiss PMQII spectrophotometer or a Hitachi-Perkin-Elmer 139 spectrophotometer.

Amino acid analysis

The procedure followed was essentially that described in the Technicon manual which is based on the method of Moore & Stein (1954a,b).

The following minor modifications, which will be described below, were made. Helium gas rathar than nitrogen was used to provide an oxygen-free atmosphere. In filling the outograd 80 ml of the buffer was put in each chamber, and finally phenol (0.1%) was added to the citrate buffer to protect them against bacteria.

The following solutions were needed:

Sodium acetate buffer 4.0N, pH 5.5. Anhydrous sodium acetate (820 g) was added slowly to approximately 1.5 liters of water. The solution was agitated with a magnetic stirrer. The solution was cooled to room temperature. Glacial acetic acid (250 ml) was added and the solution was diluted with water to 2500 ml.

Ninhydrin solution was prepared in eight liter batches in a dark 10 liter round bottomed flask. Oxitol (1300 ml) was placed in the flask and helium was bubbled for half an hour. Ninhydrin (40 g) and hydrindantin (3 g) were added to the oxitol with magnetic stirring, helium was bubbled for half an hour. Finally sodium acetate buffer pH 5.5 (700 ml) and 6 liters diluent (1:1 water and oxitol) were added, helium was bubbled for two hours. The inlet and outlet of helium gas and outlet of ninhydrin solution were kept closed except during the runs.

Citrate buffer pH 2.875 was prepared by dissolving sodium citrate $.5\frac{1}{2}\ H_2O$ (17.81 g; 0.15N with respect to Na) in 900 ml of water. 2.00N NaOH (25 ml) and thiodiglycol (5 ml) were added to the solution and the solution was shaked well. Brij solution (10 ml) containing 0.1% phenol was added and the solution was titrated with 6.0N HCl to pH 2.875 using a previously standardized pH meter (Radiometer, pH-meter-4, type RH-M_4C). The solution was finally diluted to one liter with water and

the pH was adjusted if necessary.

Citrate buffer pH 3.80 was prepared in the same manner as the citrate buffer pH 2.875. The solution was titrated with 6N HCl to pH 3.80.

Citrate buffer pH 5.00 was prepared by dissolving sodium citrate .5½ H_2O (17.81 g; 0.15N with respect to Na) in 900 ml of water. 2.00N NaOH (25 ml) was added to the solution. Sodium chloride (35.07 g) was dissolved in the solution. Brij solution (10 ml) was added and the solution was titrated with 6N HCl to pH 5.00. Finally the solution was diluted to one liter with water.

Carbonate buffer pH 10.0 was prepared by mixing 183.3 ml of sodium carbonate solution (0.2M) with 150 ml of sodium bicarbonate sol ution (0.2M) and the solution was diluted to one liter with water.

Standard glycine solution (1mM) was prepared by dissolving 7.51 mg of glycine in 0.1N HCl in a 100 ml volumetric flask.

A standard mixture of amino acids (1mM) was prepared by dissolving the amino acids in 0.1N HCl. Some methanol was added to dissolve certain amino acids. The total volume of the solution was 250 ml. Table II shows the composition of the mixture.

The preparation of DNP-amino acids derivatives was according to the procedure of Wofsy & Singer (1963). The protein (5 mg in 1 ml water) was denatured by the addition of 2 ml of ethanol and the mixture was allowed to stand for an hour at room temperature. At the end of the hour DNFB (0.1 ml) and sodium bicarbonate (0.2 g) were added. The mixture was put on a shaker for two hours at room temperature, after which the insoluble DNP-derivative was removed by

Table II

Amino Acids Composition of the Standard Amino AcidsMixture

Amino Acid	Molecular Weight	mg/250 ml
Arginine.HCl	210.7	52.67
Nor-Leucine	131.2	32.80
Aspartic acid	133.1	33.27
Threonine	119.1	29.77
Serine	105.1	26.27
Glutamic acid	147.1	36.78
Proline	115.1	28.77
Glycine	75.1	18.77
Alanine	89.1	22.27
Cystine	121.5	30.04
Valine	117.1	29.27
Methionine	149.2	37.30
Iso leucine	131.2	32.80
Leucine	131.2	32.80
Tyrosine	181.2	45.30
Phenylalanine	165.2	41.30
Lysine.HCl	182.7	45.67
Histidine.HCl	209.6	52.40

centrifuging and washed twice with water, ethanol and ether.

Myoglobin and DNP-myoglobin hydrolysates were prepared according to the method of Moore & Stein (1963) with some modifications. DNP-myoglobin (5 mg) was placed in a heavy walled pyrex test tube and suspended in 1 ml of 4N HCl (6N in the case of Mb). The mixture was left to freeze in liquid air. The test tube was evacuated and sealed under vacuum (pressure 0.025 mm Hg) using the oxygen flame while the mixture was still frozen. The test tube was allowed to warm up to room temperature and was put in an oven at $110^{\circ} \pm 1$. The hydrolysis was allowed to proceed for 24 hours (24 and 72 hours in the case of Mb). At the end of the hydrolysis time the test tube was allowed to cool and its tip was removed with a file. The excess HCl was removed under vacuum after the solution had been frozen in liquid air. The lyophilized hydrolysates were stored at 0°. Under the conditionsof hydrolysis the DNP-lysine was stable.

Amino acid analyzer. Details of the equipment and procedure are described in the manual of the Technicon amino acid analyzer. See also discussion by M. Beudjekian (Thesis, 1968).

A glass column 140 cm x 0.64 cm diameter, thermostated at 60° was packed with chromo beads type A (Technicon Chemical Company, Chauncey, New York) using citrate buffer pH 2.875. The height of the packing was 125 - 130 cm. The inlet of the column was attached to the Milton Roy Pump and the pressure guage. The pump was adjusted so that the flow rate was 0.50 - 0.51 ml/minute and the pressure was 100 - 200 p.s.i.

The sample for analysis was prepared by mixing 2.5 ml of 1mM amino acid synthetic mixture and 6 ml of 1mM DNP-lysine. The resulting solution

was diluted to 10 ml with 1:1 (v/v) mixture of 0.1N HCl and 40% sucrose. In the case of the hydrolysates the sample was prepared by dissolving the lyophilized hydrolysate in 5 ml of a 1:1 (v/v) mixture of 0.1N HCl and 40% sucrose. The sample (0.5 ml) was applied to the column with an Agla micrometer syringe fitted with a thin plastic tubing. The elution started when the buffer line to the inlet of the column was switched to the autograd which was filled according to (Table III).

The ninhydrin color values were obtained as peaks on the moving chart of the recorder of the amino acid analyzer, using the 570 mu filter. The buffer in the autograd was enough for 19 - 20 hours, which was sufficient for the elution of all amino acids. For the elution of the DNP-lysine an additional 100 ml of carbonate buffer pH 10.0 was filled in the outograd at the end of the 20 hours. The total time of each run was about 24 hours, after which the outlet of the column was disconnected from the proportioning pump. The column was washed with 0.2N NaOH for half an hour and with citrate buffer pH 2.875 for 6 hours. The ninhydrin line was washed with 0.5N NaOH for 20 minutes, detergent for 20 minutes and finally water for at least 3 hours.

The standard glycine solution was used at the beginning of every run to check the color equivalent of the ninhydrin solution. In case of large changes in the color equivalent, the ninhydrin solution was discarded. The standard glycine solution was used also to verify Beer-Lambert law hence checking the colorimeter of the auto-analyzer. Fig. 1 shows one of the experiments performed to verify the law.

The standard synthetic mixture of amino acids was used to check the reproducibility of the analyzer (Table IV) and to standardize the

Table III

Buffer Gradient

Chember	Citrate buffer pH 2.875 (ml)	Citrate buffer pH 3.80 (ml)	Citrate buffer pH 5.00 (ml)
1	80	-	-
2	80	-	-
3	80	-	-
4	80	-	-
5	-	70	10
6	6	9	65
7	-	-	80
8	-	-	80
9	-	-	80

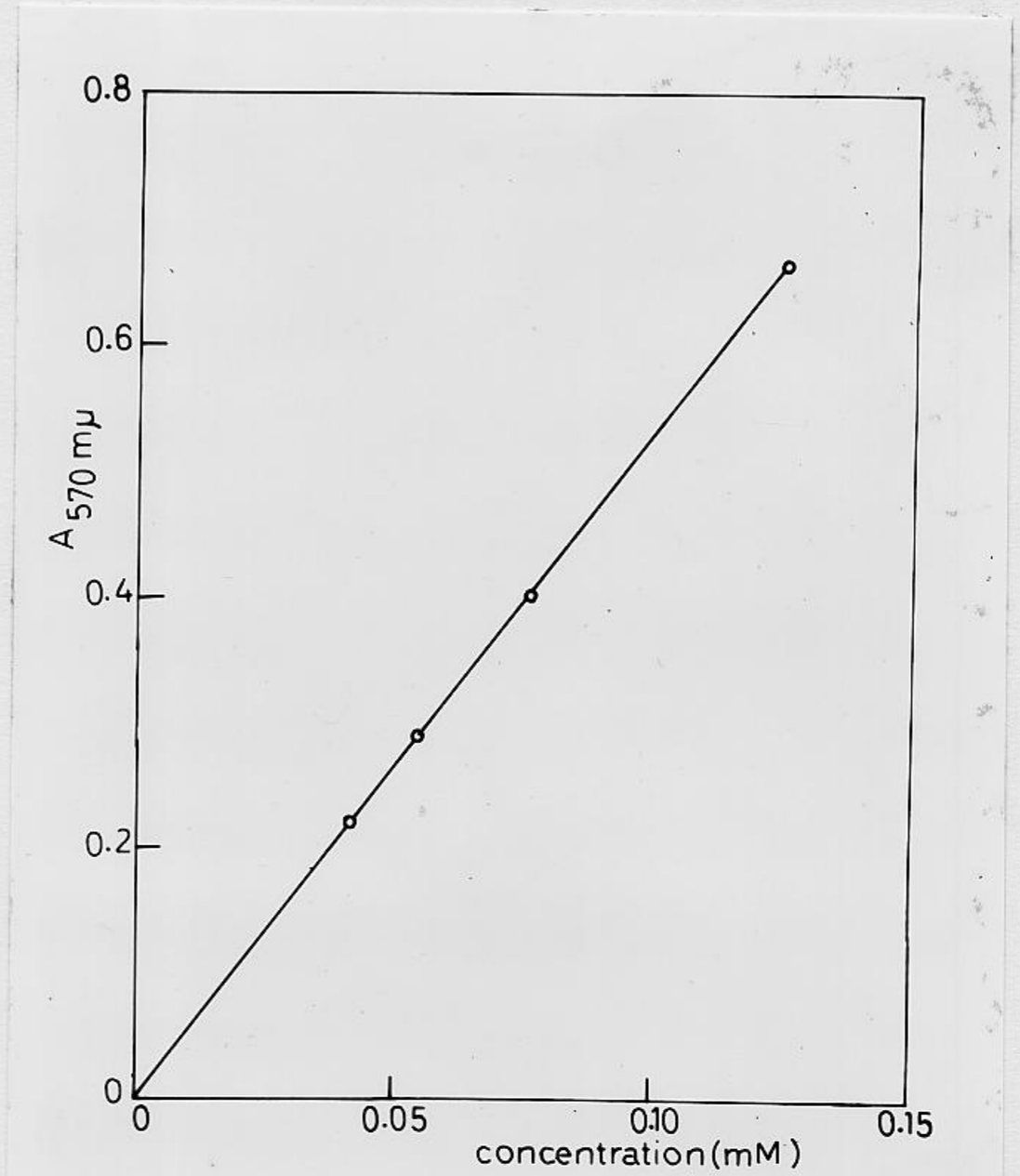

Figure 1 - Verification of Beer-Lambert law.

Table IV

Ninhydrin Color Equivalents of Amino Acids Obtained in the Technicon Amino Acid Analyzer

Amino Acid	Area* of the peak, Run A	Area* of the peak, Run B	|△Area|	%△Area
Asp	0.361	0.357	0.004	1.1
Thr	0.454	0.459	0.005	1.1
Ser	0.460	0.463	0.003	0.65
Pro	0.360	0.368	0.008	2.2
Glu	0.667	0.665	0.002	0.30
Gly	0.368	0.370	0.002	0.54
Ala	0.362	0.366	0.004	1.1
Val	0.333	0.334	0.001	0.30
Met	0.202	0.204	0.002	0.99
Leu	0.535	0.535	0.000	0
Nor-Leu	0.471	0.476	0.005	1.1
Tyr	0.485	0.490	0.005	1.0
Phe	0.476	0.489	0.013	2.8
Lys	0.490	0.485	0.005	1.0
His	0.695	0.700	0.005	0.72
Arg	0.476	0.476	0.000	0

* Area has arbitrary units which is equal to Absorbance x Inches and it is related to the amount of the amino acid analyzed.

batches of ninhydrin solution. The areas of the peaks representing the ninhydrin color equivalent, shown in Table IV were calculated according to the equation:

$$\text{Area} = \tfrac{1}{2} \text{ maximum } A_{570m\mu} \times \text{Width at half maximum } A_{570m\mu} \text{ *}$$

Titrations

The samples of myoglobins to be titrated were prepared in CO_2-free water. The concentration was around 2×10^{-3}M. The sampleswere titrated after being passed through a Dintzis or a Sephadex G-25 column. The sample applied to the column was 1 ml in the case of the Dintzis column and 0.5 ml in the case of the Sephadex column. The elution volume was 5 - 10 ml. The isoionic points were obtained by measuring the pH of the eluted sample. Titrations with 0.452N HCl and 0.374N NaOH were performed with an Agla micrometer syringe. A Radiometer, pH-meter-4, type RH-M_4C was used for pH measurements during titrations. Titrations were carried in a CO_2-free atmosphere by passing helium through the solution. The solution in the titration cell was stirred magnetically or by the passage of helium through the solution. The same titrations were performed on 10 ml of water eluted from the column. The titration values obtained for the myoglobin were corrected with respect to those obtained for water.

Dintzis column. The different cycles of the Dintzis column were prepared as follows:

1. H^+ cycle: The cation exchange resin (IR-120, 20 - 50 mesh. Purchased as the Na^+ cycle) was converted to the H^+ cycle by

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* The correct equation is: A = height at maximum x width at half maximum, but as seen in the equation for calculating the number of residues on page 28 the error will cancel out, since relative values are calculated.

passing a 50 fold excess of 6N H_2SO_4 over the Na^+ cycle in a column. The resin was washed with CO_2-free water until the effluent had pH 6.0.

2. NH_4^+ cycle: The cation exchange resin was converted to the NH_4^+ cycle by passing a 10 fold excess of 3N NH_4Cl over the Na^+ in a column.

3. ^-OH cycle: The anion exchange resin (IRA-400, 20 - 50 mesh, purchased as Cl^- cycle) was converted to ^-OH cycle by passing a 50 fold excess of 3N carbonate-free NaOH over the Cl^- cycle in a column. The resin was washed several times with CO_2-free water and stored in a closed bottle under CO_2-free water.

4. Ac^- cycle: The anion exchange resin was converted to the acetate cycle by passing a 10 fold excess of 3N sodium acetate over Cl^- cycle in a column.

The different cycles were packed in a glass column of 2 cm diameter in the following sequence from top to bottom, NH_4^+ cycle (1 cm), Ac^- cycle (1 cm), $H^+ + {}^-OH$ cycle (20 cm) and H^+ cycle (1 cm). The inlet of the column was connected to a CO_2-free water reservoir and the outlet to the titration cell. The sample took about 20 minutes to pass through the column and the column was washed exhaustively with CO_2-free water before applying another sample.

Sephadex G-25 column. A glass chromatographic column, 30 cm x 2.54 cm diam., was packed with Sephadex G-25 using CO_2-free water. The gel column was 10 cm. The sample took one hour to pass through the column and the column was washed overnight with CO_2-free water after the elution of the sample.

RESULTS

Amino acid analysis. The number of residues of a particular amino acid in the myoglobin hydrolysates was calculated according to the equation,

$$\text{number of residues} = \frac{S_{Arg}}{S_{aa}} \cdot \frac{A_{aa}}{A_{Arg}} \cdot N_{Arg},$$

where,

S_{Arg} is the area of the Arg peak in the run using the synthetic mixture of amino acids,

S_{aa} the area of the amino acid peak in question in the run using the synthetic mixture,

A_{Arg} the areaof the Arg peak in the run of myoglobin hydrolysate,

A_{aa} the area of the amino acid peak in question in the hydrolysate run;

N_{Arg} is the literature value for the number of Arg residues in myoglobin and has a value of 4 in sperm whale Mb or 2 in horse Mb.

Arginine was chosen as the internal standard, because of the consistent results obtained between the runs.

As shown in Table V, the number of residues of amino acids obtained experimentally are in agreement with those reported in literature. In DNP-derivative hydrolysate, the number of amino acid residues did not change except for lysine, where the number of residues

Table V

Number of Amino Acid Residues Found by Analysis of Native and DNP-native Myoglobins

	Sperm Whale				Horse	
Amino Acid	n(Mb)	DNP-Mb	Literature value*		DNP-Mb	Literature value+
Asp	8.4	8.9	8.2	8	12.4	11
Gly	15.9	13.0	13.6	11	15.2	16
Ala	18.7	16.0	16.6	17	12.5	16
Val	6.8	7.8	6.8	8	7.8	8
Leu	17.3	18.0	17.1	18	14.0	18
Lys	20.0	19.3	1.3	19	0.7	19
Arg	Assumed to be 4		Assumed to be 4	4	Assumed to be 2	2

* (Edmundson, 1965)

\+ (Akeson & Theorell, 1960)

dropped from 19 to 1.3 (6.8%) in the case of sperm whale and to 0.7 (3.6%) in the case of horse myoglobin. This can be the number of lysine residues that has not reacted with DNFB or the number of DNP-lysine derivative that has hydrolyzed during the time of hydrolysis of the DNP-Mb derivative. The degree of modification (Table VI) was obtained by treating the modified myoglobin with DNFB to block the unmodified lysine residues, followed by hydrolysis and amino acid analysis. Under the conditions of hydrolysis (4N HCl for 24 hours under vacuum and 110°) the DNP-lysine is stable while Ac-lysine or Gn-lysine will hydrolyze; therefore, the number of lysine residues calculated by amino acid analysis is the number of lysine residues that has been acetylated or guanidinated.

CM-Sephadex C-50 chromatography. Sperm whale (n) Mb and Ac-Mb were passed separately through the CM-column (Fig. 2a, b). The elution volume of Ac-Mb was 14 ml where as that of (n) Mb IV was 91 ml. Furthermore, the same elution volumes were observed when a mixture of 1:4 ratio of sperm whale (n) Mb and Ac-Mb was passed through the column (Fig. 2c). A clear difference in the elution volumes of horse (n) Mb (41 ml) and horse Ac-Mb (14 ml) was also observed (Fig. 3a, b).

Titrations. The native and modified myoglobin were titrated with standard 0.452N HCl and 0.374N NaOH. The starting points of the titrations were the isoionic points of the myoglobins which are listed in Table VII. The concentration of myoglobin was determined by iron analysis (Beudjekian, Thesis, 1968). The number of μequivalents (x') of acid or base needed to titrate the myoglobin to a certain pH was

Table VI

Number of Lysine Residues Obtained by Amino Acid Analysis After Treating the Modified Myoglobins with DNFB

Species	Chem. Mod.	No. of lys in native Mb	No. of lys modified	% lys modified
Sperm whale Mb	Ac (batch 1)*	19	20	105
	Ac (batch 2)		19.3	102
	Gn (supernatant)		1.2	6.3
	Gn (precipitate)		1.1	5.8
Horse Mb	Ac*	19	15.7	83
	Gn (supernatant)		1.1	5.8
	Gn (precipitate)		0.7	3.9

* used for titrations.

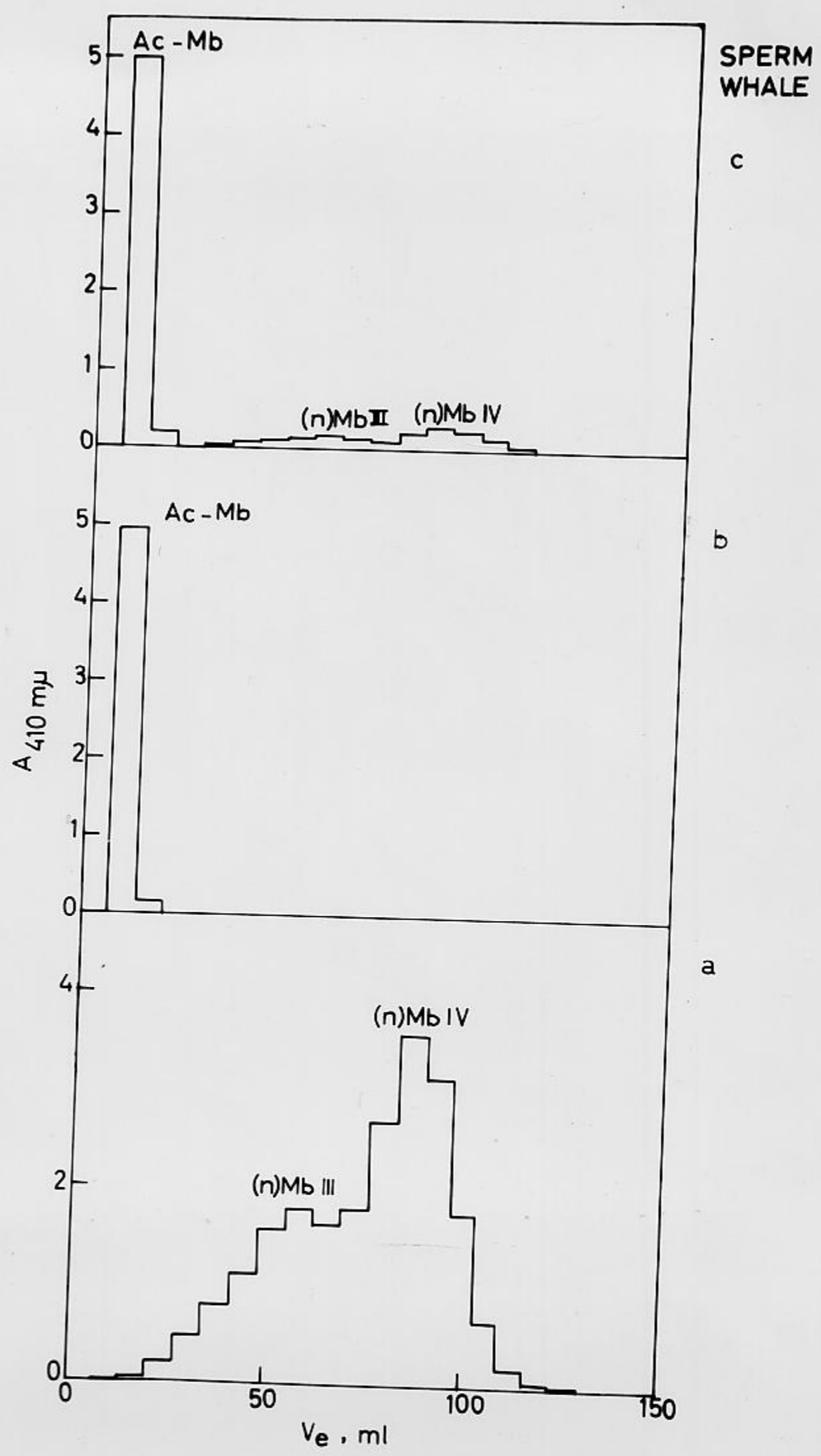

Figure 2 - Elution profile of (a): Sperm whale (n) Mb; (b) Sperm whale Ac-Mb; (c) A mixture of 1:4 ratio of sperm whale (n) Mb and its acetylated derivative.

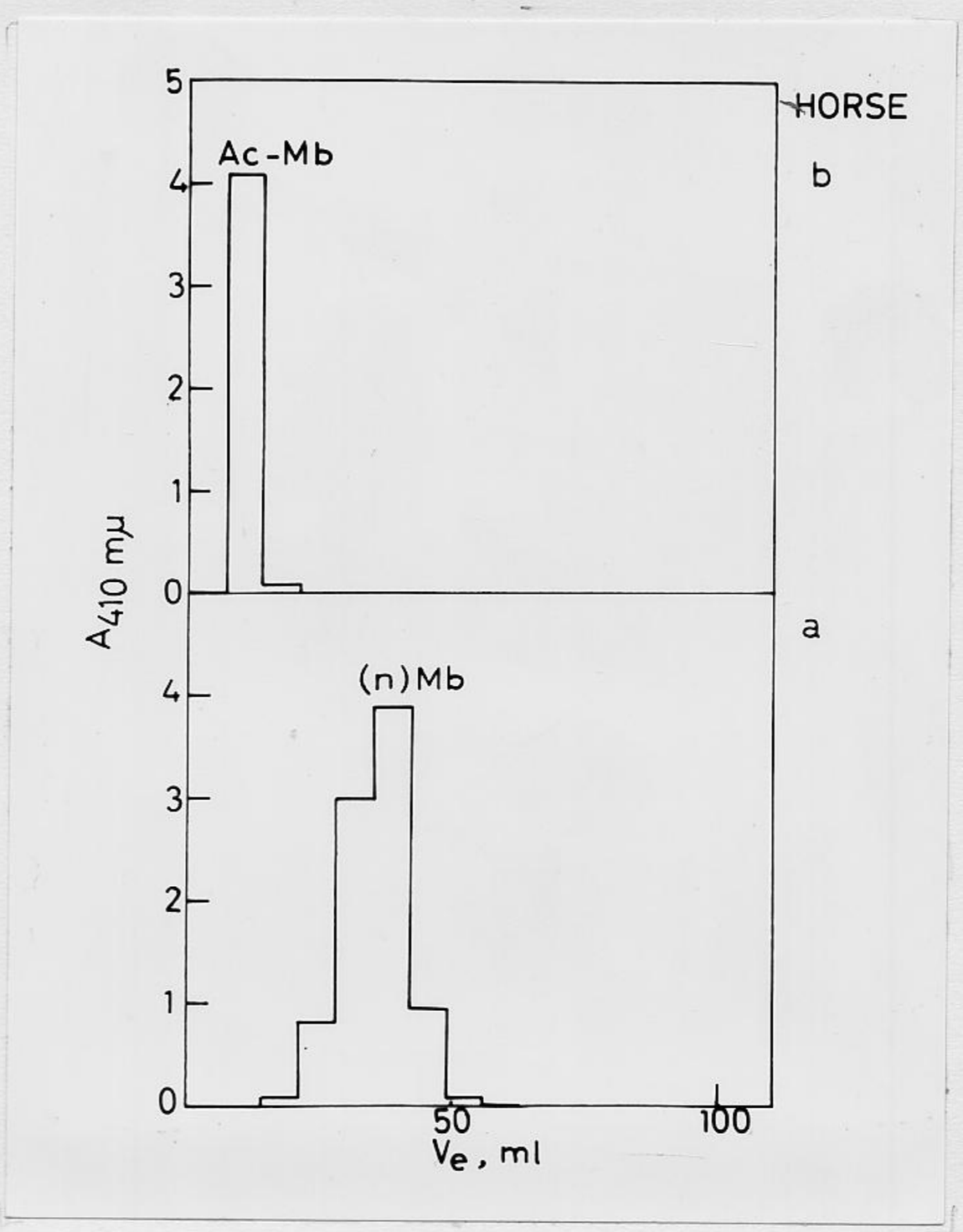

Figure 3 - Elution profile of (a) Horse (n) Mb;

(b) Horse Ac-Mb.

Table VII

Isoionic Points of Native and Modified Myoglobins

Species	Chem. modified derivative	pI
Sperm whale	(n)Mb	8.07 ± 0.01
	Ac-Mb	6.36 ± 0.06
	Suc-Mb	5.96 ± 0.06
Horse	(n)Mb	7.44 ± 0.01
	Ac-Mb	5.47 ± 0.01

corrected by subtracting the number of μequivalents of acid or base needed to titrate the same volume of water at the same pH. The corrected values are tabulated in the column headed x. In the back titrations the corrected number y (μequivalent) was obtained by subtracting the values for water from a - x (μequivalent of acid or base), as in the case of x. The titration curve of 10 ml of water (Fig. 4) was obtained by plotting μequivalents of acid or base against pH. The titration curves of myoglobins (Figs. 5 - 9) were obtained by plotting ν_{H^+} against pH, where ν_{H^+} is the number of μequivalents of acid or base added per mole of myoglobin. Titration curves of sperm whale (n)Mb (Fig. 5) and horse (n)Mb (Fig. 8) show a sharp break between pH 3.5 and 4. This break is not noticed in the back titration of the proteins. It is also not observed in any titration curve of the modified myoglobins (Figs. 6, 7 & 9). The shape of the titration curves of modified myoglobins is completely different from that of the native myoglobins in the pH range 8 - 11. In (Fig. 7) the titration curves of Suc-Mb have different scales. One of the titration curves starts from the isoionic point (pH 6.01), the other is the titration with acid of Suc-Mb after dissolving a sample in water (pH 6.95).

The detailed numerical values of titrations are given in the Appendix in Tables VIII - XII.

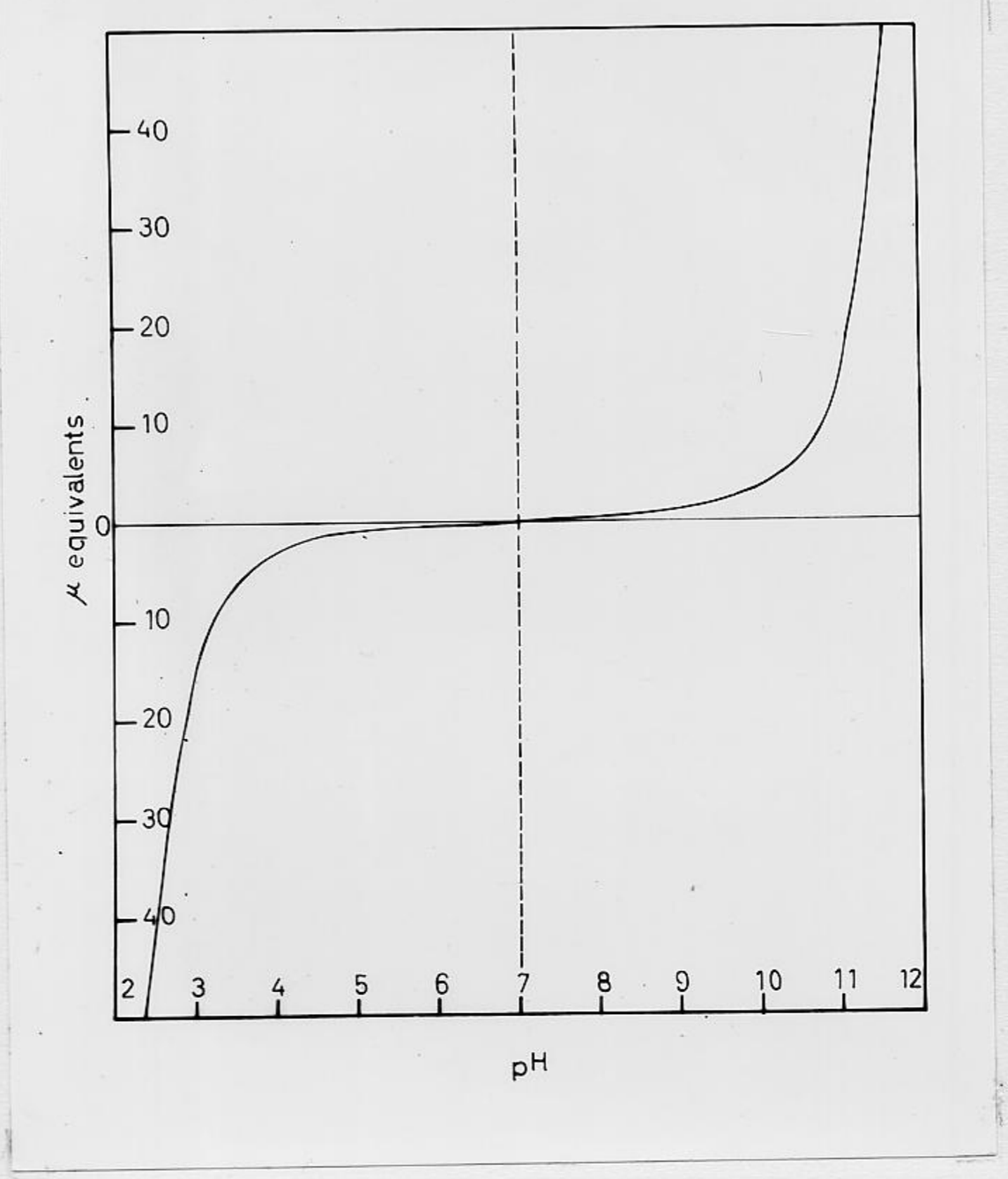

Figure 4 - Titration curve of 10 ml water.

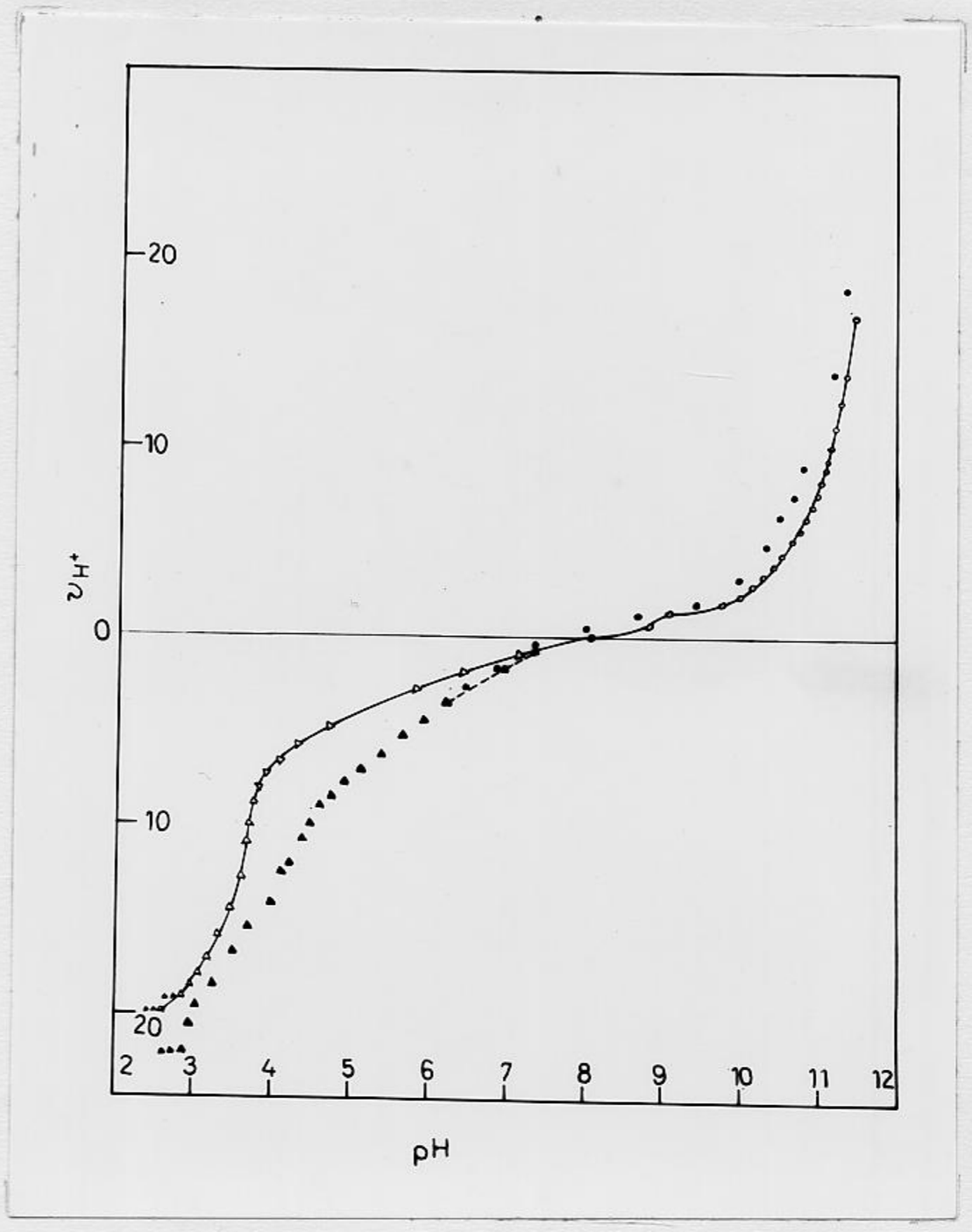

Figure 5 - Titration curves of sperm whale (n) Mb.

o—o—o : Titration with 0.374N NaOH.

• • • : Back titration with 0.452N HCl.

----- : Precipitate.

△—△—△: Titration with 0.452N HCl.

▲ ▲ ▲: Back titration with 0.374N NaOH.

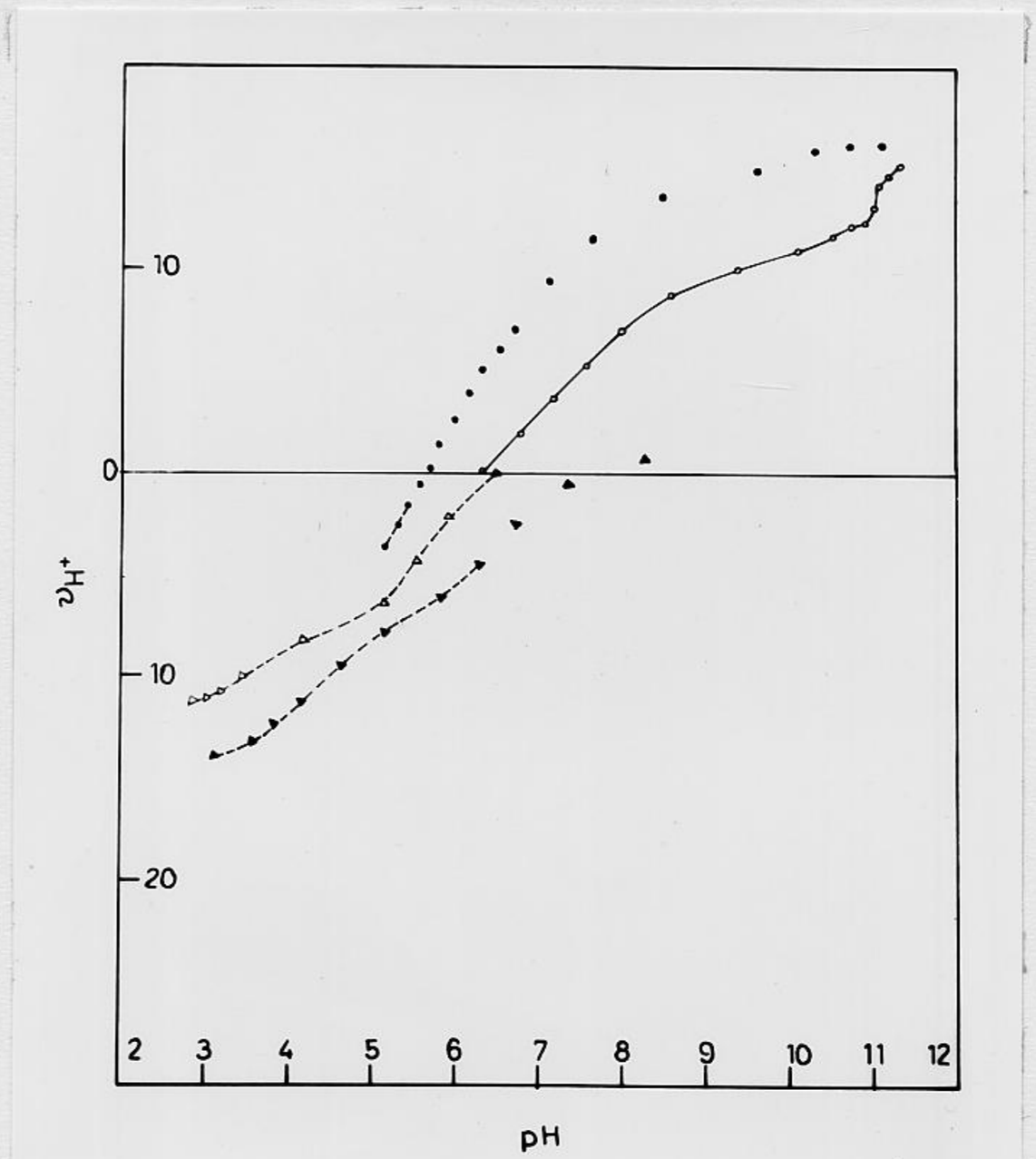

Figure 6 - Titration curves of sperm whale Ac-Mb.

o—o—o :Titration with 0.374N NaOH.

• • • :Back titration with 0.452N HCl.

----- :Precipitate.

△ - △ - △ :Titration with 0.452N HCl.

▲ ▲ ▲ :Back titration with 0.374N NaOH.

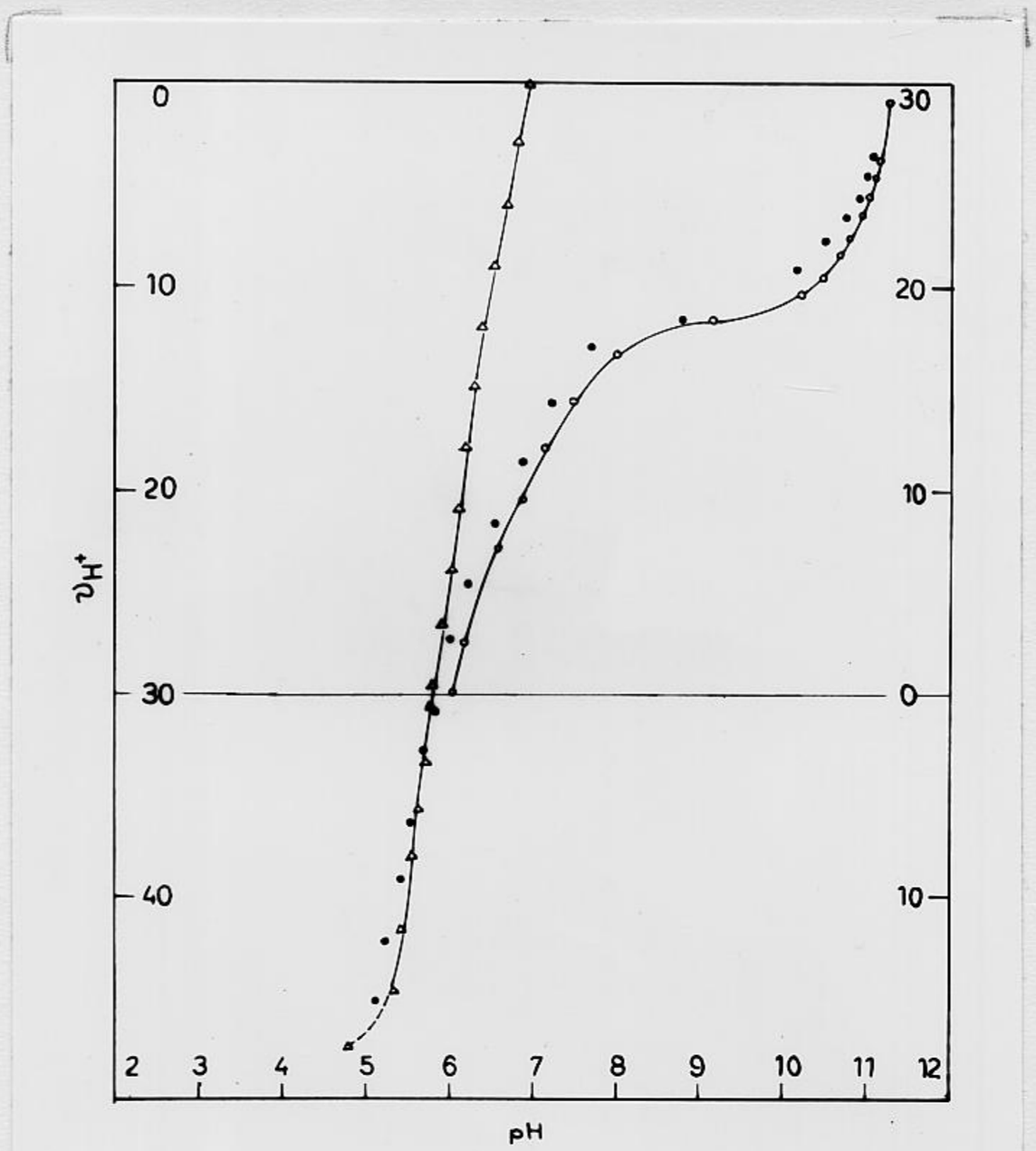

Figure 7 - Titration curves of sperm whale Suc-Mb.

o—o—o : Titration with 0.374N NaOH.

• • • : Back titration with 0.452N HCl.

----- : Precipitate.

△-△-△ : Titration with 0.452N HCl.

▲ ▲ ▲ : Back titration with 0.374N NaOH.

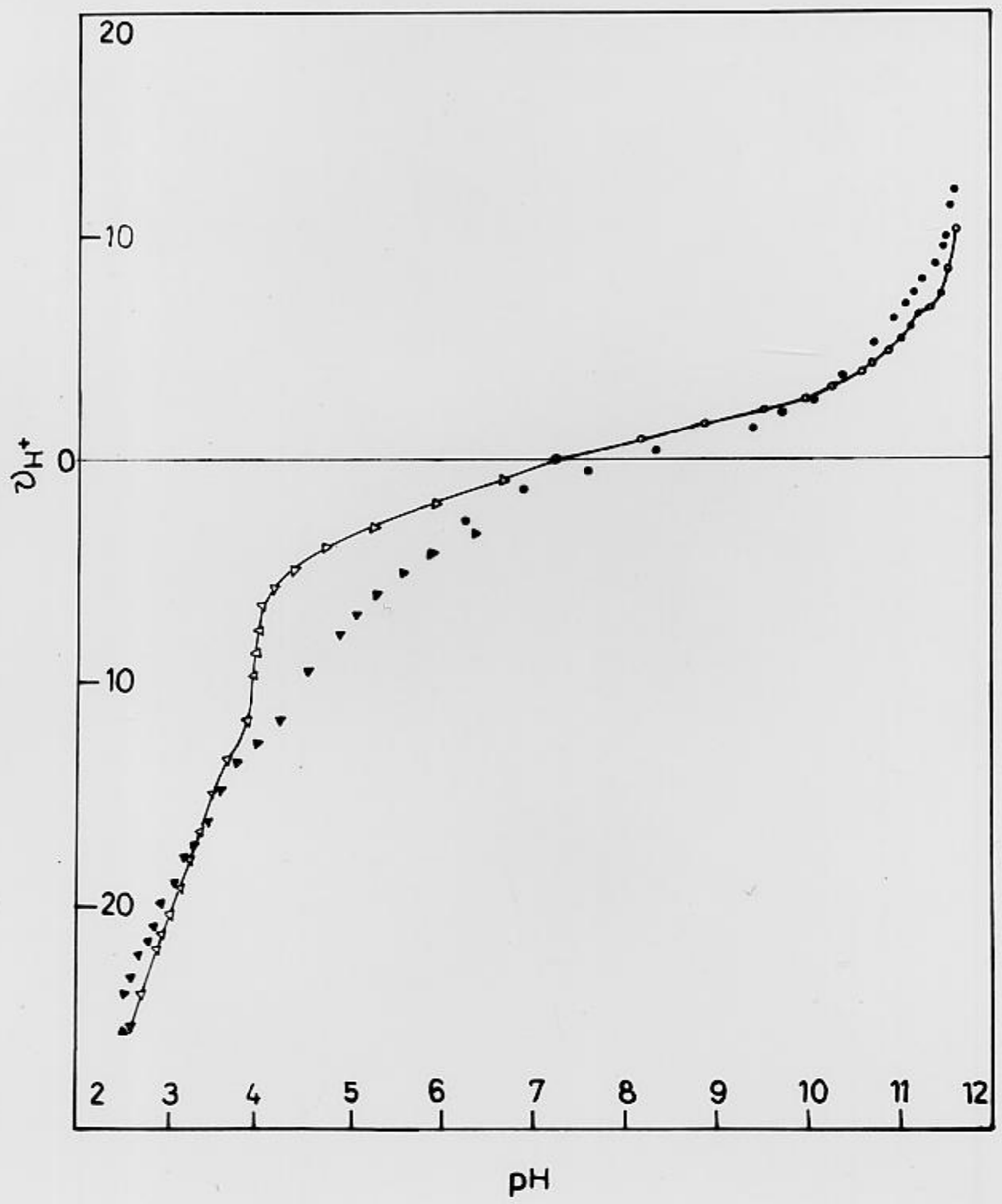

Figure 8 - Titration curves of horse (n) Mb.

o—o—o : Titration with 0.374N NaOH.

• • • : Back titration with 0.452N HCl.

----- : Precipitate.

Δ-Δ-Δ : Titration with 0.452N HCl.

▲ ▲ ▲ : Back titration with 0.374N NaOH.

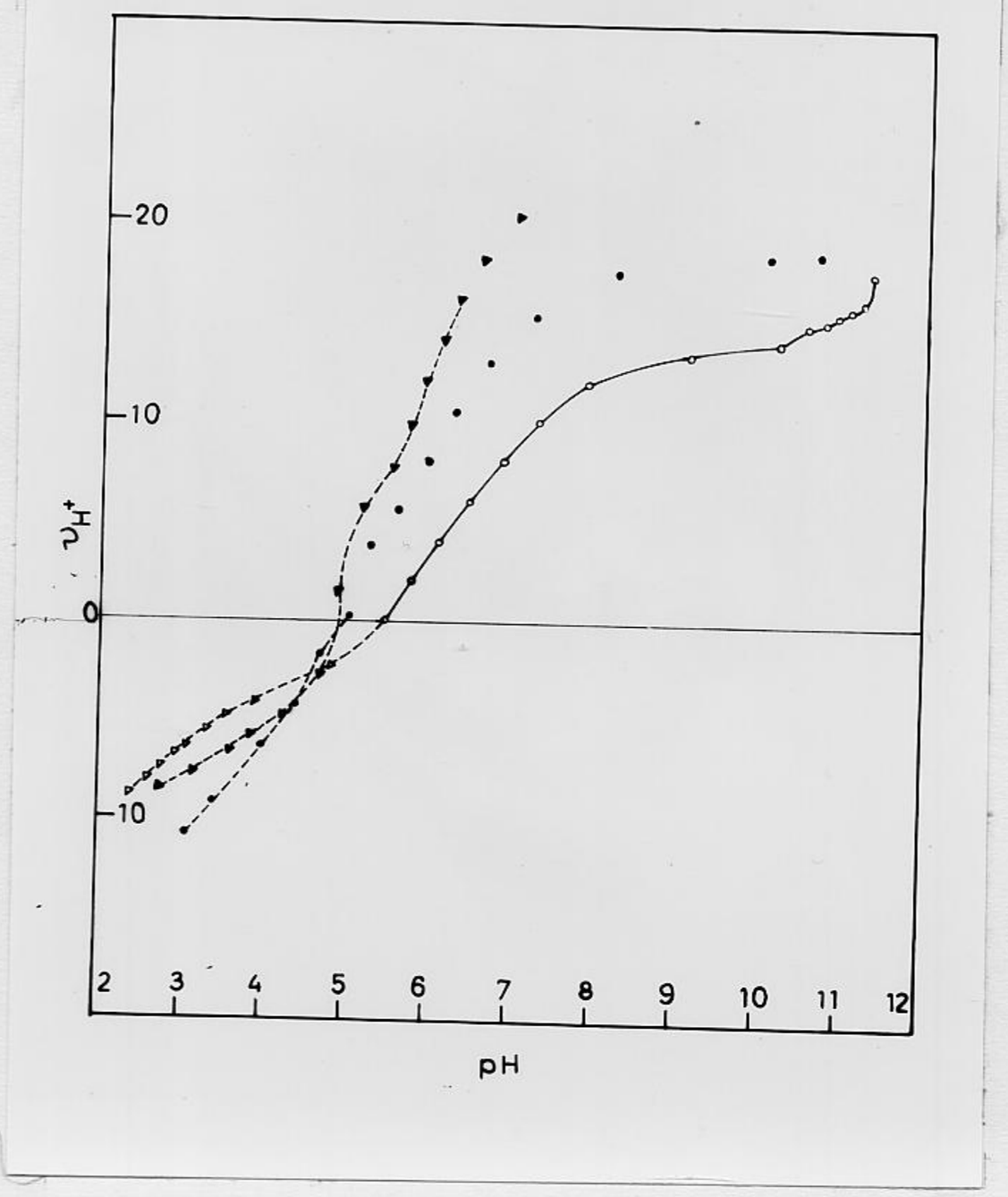

Figure 9 - Titration curves of horse Ac-Mb.

o—o—o : Titration with 0.374N NaOH.

• • • : Back titration with 0.452N HCl.

----- : Precipitate.

Δ-Δ-Δ : Titration with 0.452N HCl.

▲ ▲ ▲ : Back titration with 0.374N NaOH.

DISCUSSION

The molecular conformation of a protein molecule depends on its amino acid sequence (primary structure) and on the stabilizing effects of the non-covalent bonds (H-bonding, hydrophobic and electrostatic interactions). Chemical modification of a protein introduces covalently bonded groups in certain amino acid side chains without rupturing the primary chemical bonds in the peptide sequence. This will modify the intramolecular interactions, resulting in the reorganization of the molecular conformation. The extent of reorganization depends on the characteristics of the group introduced. The conformational reorganization in amidination and guanidination occurs without change in the net charge of the protein; the changes in conformation might be attributed to the reorganization of the non-covalent bonds that stabilize the protein. In the case of acetylation and succinylation, the net charge of the protein is changed and the conformational reorganization is caused mainly by electrostatic changes.

On acetylation, acetic anhydride attacks the ϵ -amino groups of lysine residues in myoglobin forming a covalent amide linkage and changing the positive charge on the ϵ -amino groups to zero charge. The acetylated derivative will then be a more negative protein than the native myoglobin and is expected to have a smaller elution volume than the native protein, when passed through a cation exchange column. When sperm whale (n) Mb and its acetylated

derivative were passed separately through a CM-Sephadex column, a large difference was observed in their elution volume (Fig. 2a, b), indicating a clear difference in the net charge of these proteins. The acetyl derivative can be separated from the native myoglobin, even if the native is present in small amounts (Fig. 2,c). Similarly a large difference was observed in the elution volume of horse (n) Mb and its acetylated derivative (Fig. 3a,b). Native horse myoglobin has a smaller elution volume than that of native sperm whale myoglobin. Since the number of lysine residues is the same in both myoglobins, it follows that horse myoglobin is a more negative protein, as has been demonstrated by CM-Sephadex chromatography (Awad & Kotite, 1966).

Succinylation will lower the isoionic point of myoglobin more than acetylation. As expected, the order of isoionic points of sperm whale myoglobin and its derivatives is Suc-Mb $<$ Ac-Mb $<$ (n) Mb, as shown in Table VII. Similarly the isoionic point of horse Ac-Mb is less than that of horse (n) Mb (Table VII).

Breslow & Gurd (1962) reported that 6 imidazole groups are masked in sperm whale (n) Mb and they are released on acid denaturation in their basic form. These 6 imidazole groups titrate simultaneously causing a sharp break between pH 4 and 4.5 in the forward titration with acid. The sharp break was not observed in the back titration. We observed the same sharp break between pH 4 and 3.5 in sperm whale (n) Mb (Fig. 5) and horse (n) Mb (Fig. 8) in the forward titration with acid, also the absence of a sharp break in the back titration. The sharp break was not observed in any of the modified myoglobins (Figs. 6, 7 & 9). This indicates the unfolding of the myoglobin molecule upon

acetylation and succinylation. It was practically impossible to titrate the sperm whale Suc-Mb with HCl starting at the isoionic point (pH 6.01). Precipitation occurred at the isoionic point and coagulation was observed during the titration. However, the Suc-Mb was soluble above the isoionic point, so that it was possible to titrate with NaOH starting at the isoionic point. A sample of sperm whale Suc-Mb was titrated with HCl after dissolving in water (pH 6.95) to get the acidic titration of Suc-Mb (Fig. 7). Sperm whale Ac-Mb precipitated at the isoionic point (pH 6.3), but was soluble at higher pH as well as at low pH (pH 3.5) (Fig. 6). Horse Ac-Mb precipitated also at the isoionic point (pH 5.47) and it was insoluble at low pH but soluble above the isoionic point (Fig. 9).

The titration curve with base of sperm whale (n) Mb (Fig. 5) shows the titration of two groups between pH 8.5 and 9.5 which might be the N-terminal group and the $Fe(H_2O)^+$ group; it shows also the titration of one group at pH 11.0 which could be, according to Hermans (1962), one of the tyrosine residues. The basic titration curve of sperm whale Ac-Mb (Fig. 6) shows the titration of 8 groups between pH 6.3 and 8.5 which might be imidazole groups, the other four imidazoles could be the four groups titrating between pH 6.3 and 5 in the acidic titration curve. The basic titration curve does not show the titration of lysine residues as was seen above pH 10 in the titration curve of native myoglobin (Fig. 5). The titration of the N-terminal group and the $Fe(H_2O)^+$ group in the Ac-Mb is not as obvious as in the case of (n) Mb. It can be seen that at least one group is titrating between pH 10.5 and 11 in Ac-Mb which is most

probably one of the tyrosine groups. In the titration with acid of sperm whale Suc-Mb (Fig. 7) there are 48 groups titrating in the pH range 6.95 - 4.5. These groups might be 6 imidazole groups between pH 6.95 and 6.70; the remaining 42 groups might be the free carboxyls of succinyl groups (19), the free carboxyl groups of glutamic acid (14), the free carboxyl groups of aspartic acid (8) and the C-terminal carboxyl. The basic titration curve of Suc-Mb (Fig. 7) shows the titration of 17 groups in the pH range 6 - 8; 12 of these groups might be the imidazole groups while the other 5 groups could be protonated free carboxyls of succinyl groups. The titration of lysine residues is also not seen.

The basic titration of horse (n) Mb (Fig. 8) is almost identical with that of the sperm whale (n) Mb. Between pH 5.3 and 8 in the basic titration curve of horse Ac-Mb (Fig. 9) there are 12 groups titrating which are most probably the 12 imidazole groups of the histidine residues. The basic titration curve of horse Ac-Mb does not show the titration of lysine residues above pH 10.0 as in the native horse Mb. There is one group titrating at pH 10.5 which could be one of the tyrosine residues.

It seems that acetylation and succinylation of sperm whale Mb and acetylation of horse Mb result in the unfolding of the myoglobin molecule, exposing all the titratable groups to the solvent. The unfolding of the molecule might be mainly due to intramolecular electrostatic repulsion. It might be also due to rupturing of hydrogen bonds. It seems also that the effect of succinylation on the unfolding of the molecule is greater than that of acetylation.

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APPENDIX

Table VIII

Titration of Sperm Whale Myoglobin

(a) Titration with 0.374N NaOH

pI : 8.07

Mb conc. : 2.03×10^{-3}M

Vol. of sample applied to column: 1 ml

Titration volume : 10 ml

Vol (ml)	x' (µeq)	x	ν_{H^+}	pH
0	0	0	0	8.07
0.005	1.86	1.46	0.720	8.85
0.010	3.74	2.94	1.44	9.05
0.015	5.59	3.59	1.77	9.77
0.020	7.45	4.65	2.29	10.02
0.025	9.34	5.74	2.82	10.20
0.030	11.2	7.00	3.45	10.33
0.035	13.0	7.80	3.84	10.45
0.040	14.9	8.90	4.38	10.54
0.045	16.8	9.80	4.82	10.62
0.050	18.6	10.7	5.27	10.69
0.055	20.6	11.6	5.72	10.76
0.060	22.4	12.6	6.21	10.82
0.065	24.3	13.3	6.55	10.87
0.070	26.1	14.3	7.05	10.91

Table VIII (a) cont'd.

Vol (ml)	x' (μeq)	x	ν_{H^+}	pH
0.075	28.0	15.2	7.49	10.95
0.080	29.8	15.6	7.68	11.00
0.085	31.7	16.7	8.24	11.03
0.090	33.6	17.8	8.76	11.06
0.095	35.4	18.2	8.96	11.10
0.100	37.4	19.2	9.45	11.13
0.110	40.9	20.7	10.2	11.18
0.120	44.7	22.7	11.2	11.22
0.140	52.0	25.2	12.4	11.31
0.160	59.6	28.4	14.0	11.38
0.180	67.3	30.9	15.7	11.44
0.200	74.5	34.5	17.0	11.49
0.250	93.4	45.6	22.4	11.60
0.300	112	58.6	28.8	11.68

(b) Back titration with 0.452N HCl

a = 186 μeq.

Titration volume = 10 ml

Vol (ml)	x' (μeq)	a-x'	y	ν_{H^+}	pH
0	0	186			11.90
0.15	67.6	118	65	32.0	11.66
0.20	90.4	96	51.2	25.2	11.55

Table VIII (b) Cont'd.

Vol (ml)	x' (μeq)	a-x'	y	ν_{H^+}	pH
0.260	117	69	37.6	18.5	11.38
0.300	136	50	28.4	14.0	11.22
0.350	158	28	18.4	9.07	10.80
0.360	163	23	15.2	7.49	10.68
0.370	167	19	13.3	6.55	10.51
0.380	172	14	9.8	4.83	10.31
0.390	177	9	6.3	3.10	9.98
0.400	181	5	3.4	1.67	9.40
0.405	183	3	2.4	1.18	8.69
0.410	185	1	1	0.493	8.01
0.415	187	-1	-0.6	-0.296	7.38
0.420	190	-4	-3.4	-1.67	6.89
0.425	192	-6	-5.2	-2.56	6.49
		Precipitate			

(c) Titration with 0.452N HCl

pI : 8.07

Mb conc. 2.03×10^{-3} M

Vol of sample applied to column : 1 ml

Titration volume : 10 ml

Vol (ml)	x' (μeq)	x	ν_{H^+}	pH
0	0	0	0	8.07
0.005	2.26	1.76	0.867	7.17

Table VIII (c) Cont'd.

Vol (ml)	x' (μeq)	x	ν_{H^+}	pH
0.010	4.52	3.72	1.83	6.45
0.015	6.76	5.56	2.74	5.81
0.020	9.04	7.44	3.66	5.20
0.025	11.3	9.50	4.68	4.72
0.030	13.6	11.5	5.67	4.37
0.035	15.8	13.0	6.41	4.10
0.040	18.1	14.6	7.20	3.94
0.045	20.3	16.1	7.94	3.83
0.050	22.6	18.0	8.88	3.76
0.055	24.8	20.1	9.92	3.74
0.060	27.1	22.0	10.8	3.70
0.070	31.6	25.8	12.7	3.61
0.080	36.2	29.0	14.3	3.48
0.090	40.6	32.0	15.8	3.33
0.10	45.2	34.6	17.0	3.20
0.11	49.6	36.0	17.8	3.07
0.12	54.4	37.3	18.4	2.96
0.13	58.8	38.6	19.0	2.85
0.14	63.3	38.7	19.1	2.76
0.15	67.6	38.9	19.2	2.69
0.16	72.4	40.2	19.8	2.63
0.17	77.0	40.4	19.9	2.57
0.18	81.4	40.2	19.8	2.51
0.19	85.9	41.3	20.3	2.41

Table VIII Cont'd.

(d) Back titration with 0.374N NaOH

a = 226 μeq

Titration volume : 10 ml

Vol (ml)	x' (μeq)	$a-x'$	y	ν_{H^+}	pH
0	0	226			1.82
0.35	130	96	47	23.2	2.41
0.40	149	77	43.4	21.4	2.61
0.41	153	73	42.6	21.0	2.66
0.42	157	69	42.6	21.0	2.73
0.43	160	66	43.2	21.3	2.79
0.44	164	62	42.4	20.9	2.86
0.45	168	58	41.4	20.4	2.94
0.460	172	54	39.6	19.5	3.03
0.480	179	47	37.2	18.3	3.25
0.500	186	40	33.6	16.6	3.54
0.510	190	36	31.1	15.3	3.71
0.520	194	32	28.9	14.2	4.01
0.530	198	28	25.2	12.4	4.12
0.535	200	26	24.6	12.1	4.23
0.540	202	24	21.8	10.7	4.37
0.545	204	22	20.1	9.90	4.50
0.550	206	20	18.1	8.91	4.63
0.555	207	19	17.2	8.47	4.77
0.560	209	17	15.3	7.54	4.95
0.565	210	16	14.4	7.09	5.16
0.570	212	14	12.6	6.21	5.40
0.575	214	12	10.6	5.22	5.67
0.580	216	10	8.9	4.38	5.93
0.585	218	8	7.0	3.45	6.23
		Precipitate			
0.590	220	6	5.2	2.56	6.56
0.595	222	4	3.4	1.67	6.97
0.600	224	2	1.4	0.689	7.35

Table IX

Titration of Horse Myoglobin

(a) Titration with 0.374N NaOH

pI : 7.45

Mb conc. : 1.97×10^{-3} M

Vol. of sample applied to column : 1 ml

Titration volume: 8 ml

Vol (ml)	x' (μeq)	x	ν_{H^+}	pH
0	0	0	0	7.45
0.005	1.86	1.62	0.822	8.16
0.010	3.74	3.14	1.59	8.87
0.015	5.59	4.23	2.15	9.52
0.020	7.45	5.13	2.60	9.95
0.025	9.34	6.22	3.16	10.22
0.030	11.2	7.1	3.60	10.41
0.035	13.0	7.6	3.86	10.57
0.040	14.9	8.4	4.26	10.68
0.050	18.6	9.6	4.87	10.87
0.060	22.4	10.6	5.38	11.01
0.070	26.1	11.8	5.99	11.12
0.080	29.8	12.8	6.50	11.20
0.090	33.6	13.2	6.70	11.28
0.10	37.4	13.3	6.75	11.35
0.12	44.7	14.5	7.36	11.46
0.14	52.0	17.0	8.63	11.54
0.16	59.6	20.2	10.3	11.62

(b) Back titration with 0.452N HCl

a = 130 μeq

Titration volume: 8 ml

Vol (ml)	x' (μeq)	a-x'	y	ν_{H^+}	pH
0	0	130			11.99
0.150	67.6	62	23.8	12.1	11.60
0.160	72.4	58	22.2	11.3	11.56

Table IX (b) Cont'd.

Vol (ml)	x' (μeq)	a-x'	y	ν_{H^+}	pH
0.170	77.0	53	19.5	9.90	11.52
0.180	81.4	49	18.8	9.54	11.46
0.190	85.9	44	17.2	8.73	11.40
0.200	90.4	40	16.9	8.58	11.34
0.210	95.0	35	15.7	7.97	11.26
0.220	99.5	31	14.8	7.51	11.17
0.230	104	26	13.2	6.70	11.06
0.240	108	22	12.3	6.24	10.92
0.250	113	17	10.2	5.18	10.74
0.260	117	13	8.5	4.31	10.47
0.265	120	10	6.5	3.30	10.29
0.270	122	8	5.4	2.74	10.05
0.275	124	6	4.2	2.13	9.71
0.280	126	4	2.8	1.42	9.41
0.285	129	1	0.6	0.304	8.35
0.290	131	-1	-1	-0.507	7.57
0.295	133	-3	-2.8	-1.42	6.88
0.300	136	-6	-5.5	-2.79	6.25
		Precipitate			

(c) Titration with 0.452N HCl

pI : 7.43

Mb conc : 1.97×10^{-3} M

Vol. of sample applied to column : 1 ml

Titration volume: 10 ml.

Vol (ml)	x' (μeq)	x	ν_{H^+}	pH
0	0	0	0	7.43
0.005	2.26	1.96	0.995	6.65
0.010	4.52	3.82	1.94	5.92
0.015	6.76	5.66	2.87	5.23
0.020	9.04	7.64	3.88	4.72
0.025	11.3	9.50	4.82	4.36
0.030	13.6	11.2	5.68	4.13

Table IX (c) Cont'd.

Vol (ml)	x' (μeq)	x	ν_{H^+}	pH
0.035	15.8	13.0	6.60	4.00
0.040	18.1	15.2	7.72	3.98
0.045	20.3	17.1	8.68	3.93
0.050	22.6	19.3	9.80	3.92
0.060	27.1	23.3	11.8	3.82
0.070	31.6	26.5	13.4	3.64
0.080	36.2	29.6	15.0	3.47
0.090	40.6	32.5	16.5	3.34
0.100	45.2	35.5	18.0	3.23
0.110	49.6	37.8	19.2	3.12
0.120	54.4	40.0	20.3	3.01
0.130	58.8	41.6	21.1	2.92
0.140	63.3	42.9	21.8	2.85
0.150	67.6	44.6	22.6	2.78
0.160	72.4	46.2	23.4	2.72
0.170	77.0	47.0	23.9	2.66
0.180	81.4	48.2	24.5	2.61
0.190	85.9	49.9	25.3	2.57
0.200	90.4	50.2	25.5	2.52

(d) Back titration with 0.374N NaOH

a = 203 μeq

Titration volume : 10 ml

Vol (ml)	x' (μeq)	a-x'	y	ν_{H^+}	pH
0	0	203			1.98
0.300	112	91	48.0	24.4	2.48
0.310	117	86	46.8	23.8	2.52
0.320	119	84	46.2	23.5	2.55
0.330	123	80	45.7	23.2	2.59
0.340	127	76	44.4	22.5	2.63
0.350	130	73	43.8	22.2	2.67
0.360	134	69	42.8	21.7	2.72

Table IX (d) Cont'd.

Vol (ml)	x' (μeq)	a-x'	y	ν_{H^+}	pH
0.370	138	65	42.2	21.4	2.78
0.380	142	61	41.0	20.8	2.84
0.390	146	57	39.0	19.8	2.90
0.400	149	54	38.6	19.6	2.97
0.410	153	50	37.2	18.9	3.07
0.420	157	46	35.0	17.8	3.16
0.430	160	43	33.8	17.2	3.26
0.440	164	39	31.7	16.1	3.40
0.450	168	35	29.2	14.8	3.55
0.460	172	31	26.7	13.5	3.74
0.470	175	28	25.1	12.7	3.97
0.480	178	25	23.0	11.7	4.24
0.490	183	20	18.5	9.39	4.53
0.500	186	17	15.6	7.92	4.88
0.505	188	15	13.8	7.00	5.05
0.510	190	13	11.9	6.04	5.25
0.515	192	11	10.0	5.08	5.55
0.520	194	9	8.2	4.16	5.90
0.525	196	7	6.5	3.30	6.35
		Precipitate			

Table X

Titration of Sperm Whale Ac-Mb

(a) Titration with 0.374N NaOH

pI : 6.30

Mb conc. : 1.99×10^{-3} M

Vol. of sample applied to the column : 0.5 ml

Titration volume : 5 ml

Vol (ml)	x' (μeq)	x	ν_{H^+}	pH
0	0	0	0	6.30
0.005	1.86	1.71	1.68	6.76
0.010	3.74	3.54	3.56	7.16
0.015	5.59	5.29	5.32	7.56
0.020	7.45	7.00	7.04	7.98
0.025	9.34	8.79	8.84	8.58
0.030	11.2	10.1	10.1	9.39
0.035	13.0	10.9	11.0	10.11
0.040	14.9	11.6	11.7	10.52
0.045	16.8	12.1	12.2	10.75
0.050	18.6	12.2	12.3	10.92
0.055	20.6	13.0	13.1	11.01
0.060	22.4	14.1	14.2	11.06
0.070	26.1	14.5	14.6	11.23
0.080	29.8	15.2	15.3	11.34

(b) Back titration with 0.452N HCl

a = 29.8 μeq

Titration volume: 5 ml

Vol (ml)	x' (μeq)	a-x'	y	ν_{H^+}	pH
0	0	29.8	15.2	15.3	11.34
0.010	4.52	25.3	16.1	16.2	11.11
0.020	9.04	20.8	16.1	16.2	10.73
0.025	11.3	18.5	15.8	15.9	10.36
0.030	13.6	16.2	14.8	14.9	9.65

Table X (b) Cont'd.

Vol (ml)	x' (μeq)	a-x'	y	ν_{H^+}	pH
0.035	15.8	14.0	13.5	13.6	8.49
0.040	18.1	11.7	11.4	11.5	7.62
0.045	20.3	9.5	9.4	9.45	7.14
0.050	22.6	7.2	7.1	7.13	6.73
0.0525	23.7	6.1	6.0	6.03	6.53
0.0550	24.8	5.0	5.0	5.02	6.34
0.0575	25.9	3.9	3.9	3.91	6.17
0.0600	27.1	2.7	2.6	2.61	6.00
0.0625	28.2	1.6	1.4	1.41	5.86
0.0650	29.4	0.4	0.2	0.201	5.70
0.0675	30.5	-0.7	-0.5	-0.502	5.56
0.0700	31.6	-1.8	-1.6	-1.61	5.43
		Precipitate			
0.0725	32.7	-2.9	-2.6	-2.61	5.30
0.0750	33.8	-4.0	-3.7	-3.72	5.15
0.0800	36.2	-6.4	-6.0	-6.03	4.74
0.0900	40.6	-10.8	-9.7	-9.75	4.01
0.0950	42.9	-13.1	-11.2	-11.3	3.72
0.100	45.2	-15.4	-12.4	-12.5	3.47
0.110	49.6	-19.8	-13.8	-13.9	3.08
0.120	54.4	-24.6	-13.9	-14.0	2.85
0.130	58.8	-29.0	-14.6	-14.7	2.67

(c) Titration with 0.452N HCl

pI : 6.48

Mb conc.: 1.99×10^{-3} M

Vol. of sample applied to the column: 0.5 ml

Titration volume : 5 ml.

Vol (ml)	x' (μeq)	x	ν_{H^+}	pH
0	0	0	0	6.48
0.005	2.26	2.11	2.12	5.94
		precipitate		

Table X (c) Cont'd.

Vol (ml)	x' (μeq)	x	ν_{H^+}	pH
0.010	4.52	4.27	4.29	5.55
0.015	6.76	6.41	6.44	5.16
0.020	9.04	8.14	8.18	4.20
0.030	13.6	10.1	10.1	3.40
0.035	15.8	10.7	10.8	3.18
		Precipitate dissolved		
0.040	18.1	11.1	11.2	3.01
0.045	20.3	10.9	11.0	2.86
0.050	22.6	10.2	10.3	2.74
0.055	24.9	9.7	9.75	2.64
0.060	27.1	8.5	8.54	2.55
0.070	31.6	7.7	7.74	2.41
0.080	36.2	8.2	8.24	2.30

(d) Back Titration with 0.374N NaOH

a = 90.4 μeq

Titration volume : 5 ml

Vol (ml)	x' (μeq)	a-x'	y	ν_{H^+}	pH
0	0	90.4			1.75
0.160	59.6	30.7	11.7	11.8	2.54
0.170	63.5	26.9	12.8	12.9	2.68
0.180	67.3	23.1	11.9	12.0	2.87
0.190	71.0	19.4	13.8	13.9	3.14
0.200	74.7	15.7	13.1	13.2	3.57
		Precipitate			
0.205	76.6	13.8	12.2	12.3	3.83
0.210	78.4	12.0	11.1	11.2	4.17
0.215	80.3	10.1	9.6	9.65	4.65
0.220	82.2	8.2	7.8	7.84	5.18
0.225	84.1	6.3	6.1	6.13	5.85
0.230	85.9	4.5	4.5	4.52	6.31
		Precipitate dissolved			
0.235	87.8	2.6	2.5	2.51	6.73
0.240	89.7	0.5	0.5	0.502	7.35

Table XI

Titration of Sperm Whale Suc-Mb

(a) Titration with 0.374N NaOH

pI : 6.01

Mb conc. 1.50×10^{-3} M

Vol. of sample applied to the column : 0.5 ml

Titration volume: 5 ml

Vol (ml)	x' (μeq)	x	ν_{H^+}	pH
0	0	0	0	6.01
0.005	1.86	1.81	2.41	6.16
0.010	3.74	3.64	4.92	6.34
0.015	5.59	5.39	7.19	6.58
0.020	7.45	7.20	9.60	6.88
0.025	9.34	9.04	12.1	7.14
0.030	11.2	10.8	14.4	7.47
0.035	13.0	12.5	16.7	7.96
0.040	14.9	13.9	18.5	9.15
0.046	17.2	14.8	19.7	10.20
0.050	18.6	15.4	20.5	10.46
0.055	20.6	16.3	21.7	10.66
0.060	22.4	16.9	22.5	10.81
0.065	24.3	17.7	23.6	10.92
0.070	26.1	18.4	24.5	11.01
0.075	28.0	19.1	25.5	11.08
0.080	29.8	19.7	26.3	11.15
0.090	33.6	21.9	29.2	11.25

(b) Back titration with 0.452N HCl

a = 33.6 μeq

Titration volume: 5 ml

Vol (ml)	x' (μeq)	a-x'	y	ν_{H^+}	pH
0	0	33.6	21.9	29.2	11.25
0.010	4.52	29.1	19.9	26.5	11.10
0.015	6.76	26.8	19.1	25.5	11.01

Table XI (b) Cont'd.

Vol (ml)	x' (μeq)	a-x'	y	ν_{H^+}	pH
0.020	9.04	24.6	18.3	24.4	10.89
0.025	11.3	22.3	17.6	23.5	10.72
0.030	13.6	20.0	16.7	22.3	10.49
0.035	15.8	17.8	15.6	20.8	10.12
0.040	18.1	14.5	13.8	18.4	8.76
0.045	20.3	13.3	12.8	17.1	7.68
0.050	22.6	11.0	10.7	14.3	7.21
0.055	24.8	8.8	8.6	11.5	6.85
0.060	27.1	6.5	6.3	8.40	6.51
0.065	29.4	4.2	4.1	5.46	6.21
0.070	31.6	2.0	2.0	2.66	6.00
0.076	34.3	-0.7	-0.6	-0.800	5.79
0.080	36.2	-2.6	-2.5	-3.33	5.71
0.085	38.4	-4.8	-4.7	-6.27	5.54
0.090	40.6	-7.0	-6.8	-9.10	5.37
0.095	42.9	-9.3	-9.1	-12.1	5.24
0.100	45.2	-11.6	-11.3	-15.1	5.14
0.110	49.6	-16.0	-15.7	-20.9	5.00
0.120	54.4	-21.8	-21.5	-28.7	4.90
		Precipitate			
0.140	63.3	-29.7	-26.4	-35.2	3.40
0.150	67.6	-34.0	-28.5	-38.0	3.13

(c) Titration with 0.452N HCl

Mb Conc.: 1.50×10^{-3} M

Vol. of sample titrated: 0.5 ml

Titration volume: 5 ml

Starting point: pH 6.95

Vol (ml)	x' (μeq)	x	ν_{H^+}	pH
0	0	0	0	6.95
0.005	2.26	2.26	3.01	6.81
0.010	4.52	4.47	5.96	6.67

Table XI (c) Cont'd.

Vol (ml)	x' (μeq)	x	ν_{H^+}	pH
0.015	6.76	6.71	8.95	6.52
0.020	9.04	8.94	11.9	6.38
0.025	11.3	11.1	14.8	6.28
0.030	13.6	13.4	17.9	6.19
0.035	15.8	15.6	20.8	6.10
0.040	18.1	17.9	23.9	6.03
0.045	20.3	20.0	26.6	5.90
0.050	22.6	22.3	29.7	5.78
0.055	24.8	24.5	32.7	5.70
0.060	27.1	26.7	35.6	5.60
0.065	29.3	28.5	38.0	5.51
0.070	31.6	31.2	41.6	5.42
0.075	33.9	33.5	44.7	5.35
0.080	36.2	35.6	47.5	4.77
		Precipitate		
0.090	40.6	38.6	51.5	3.77

Table XII

Titration of Horse Ac-Mb

(a) Titration with 0.374N NaOH

pI : 5.47

Mb conc. 1.75×10^{-3} M

Vol. of sample applied to the column: 0.5 ml

Titration volume: 5 ml

Vol (ml)	x' (μeq)	x	ν_{H^+}	pH
0	0	0	0	5.47
		Precipitate		
0.005	1.86	1.76	2.01	5.80
0.010	3.74	3.54	4.04	6.10
		Precipitate dissolved		
0.015	5.59	5.29	6.04	6.45
0.020	7.45	7.05	8.05	6.87
0.025	9.34	8.84	10.1	7.29
0.030	11.2	10.5	12.0	7.86
0.035	13.0	11.9	13.6	9.13
0.040	14.9	12.4	14.2	10.21
0.045	16.8	13.1	15.0	10.55
0.050	18.6	13.2	15.1	10.78
0.055	20.6	13.7	15.6	10.94
0.060	22.4	13.9	15.9	11.06
0.065	24.3	14.0	16.0	11.16
0.070	26.1	14.2	16.2	11.23
0.075	28.0	14.6	16.7	11.29
0.080	29.8	15.4	17.6	11.32

(b) Back titration with 0.452N HCl

a = 29.8 μeq

Titration volume: 5 ml

Vol (ml)	x' (μeq)	a-x'	y	ν_{H^+}	pH
0	0	29.8	15.4	17.6	11.32
0.005	2.26	27.5	16.0	18.3	11.22

Table XII (b) Cont'd.

Vol (ml)	x' (μeq)	a-x'	y	ν_{H^+}	pH
0.010	4.52	25.3	16.0	18.3	11.10
0.015	6.76	23.0	16.1	18.4	10.93
0.020	9.04	20.8	16.4	18.7	10.66
0.025	11.3	18.5	16.2	18.5	10.07
0.030	13.6	16.2	15.5	17.7	8.23
0.035	15.8	14.0	13.5	15.4	7.24
0.040	18.1	11.7	11.4	13.0	6.68
0.045	20.3	9.5	9.3	10.6	6.28
0.050	22.6	7.2	7.1	8.11	5.97
0.055	24.8	5.0	5.0	5.71	5.60
0.060	27.1	2.7	2.7	3.08	5.26
0.065	29.4	0.4	0.3	0.342	5.01
		Precipitate			
0.070	31.6	-1.8	-1.6	-1.83	4.69
0.075	33.9	-4.1	-3.7	-4.23	4.33
0.080	36.2	-6.4	-5.5	-6.28	4.00
0.090	40.6	-10.8	-8.0	-9.14	3.44
0.100	45.2	-15.4	-9.5	-10.8	3.07
0.110	49.6	-19.8	-9.8	-11.2	2.82

(c) Titration with 0.452N HCl

pI : 5.46

Mb conc. 1.75×10^{-3} M

Vol. of sample applied to the column : 0.5 ml

Titration volume: 5 ml.

Vol (ml)	x' (μeq)	x	ν_{H^+}	pH
0	0	0	0	5.46
		Precipitate		
0.005	2.26	2.06	2.35	4.78
0.010	4.52	3.52	4.02	3.92
0.015	6.76	4.16	4.75	3.52
0.020	9.04	5.04	5.76	3.27

Table XII (c) Cont'd.

Vol (ml)	x' (μeq)	x	ν_{H^+}	pH
0.025	11.3	5.5	6.28	3.08
0.030	13.6	5.9	6.74	2.94
0.035	15.8	6.2	7.08	2.84
0.040	18.1	6.3	7.20	2.75
0.045	20.3	6.3	7.20	2.67
0.050	22.6	6.5	7.43	2.61
0.055	24.9	6.9	7.88	2.56
0.060	27.1	7.0	8.00	2.50
0.065	29.3	7.2	8.23	2.45
0.070	31.6	7.6	8.68	2.40

(d) Back titration with 0.374N NaOH

a = 45.2 μeq

Titration volume: 5 ml

Vol (ml)	x' (μeq)	a-x'	y	ν_{H^+}	pH
0	0	45.2			2.14
		Precipitate			
0.030	11.2	34.0	5.4	6.20	2.36
0.050	18.6	25.6	5.6	6.40	2.51
0.060	22.4	22.8	7.2	8.23	2.62
0.070	26.1	19.1	7.4	8.46	2.75
0.080	29.8	15.4	7.2	8.23	2.93
0.090	33.6	11.6	6.7	7.66	3.20
0.100	37.4	7.8	5.8	6.63	3.58
0.105	39.2	6.0	5.1	5.83	3.87
0.110	40.9	4.3	4.1	4.68	4.25
0.115	42.9	2.3	2.2	2.53	4.66
0.125	46.6	-1.4	-1.3	-1.48	4.92
0.135	50.4	-5.2	-5.1	-5.83	5.16
0.140	52.0	-6.8	-6.8	-7.77	5.54
0.145	54.0	-8.8	-8.7	-9.94	5.74
0.150	55.9	-10.7	-10.6	-12.1	5.93

Table XII (d) Cont'd.

Vol (ml)	x' (μeq)	a-x'	y	ν_{H^+}	pH
0.155	57.8	-12.6	-12.4	-14.2	6.13
0.160	59.6	-14.4	-14.2	-16.2	6.30
		Precipitate dissolved			
0.165	61.5	-16.3	-16.0	-18.3	6.59
0.170	63.4	-18.2	-17.8	-20.3	7.00
0.175	65.2	-20.0	-19.5	-22.3	7.11
0.180	67.1	-22.1	-21.6	-24.7	7.36
0.190	71.0	-25.8	-25.2	-28.8	7.65