# CHEMICALLY MODIFIED MYOGLOBIN:

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# AND COVALENTLY-LINKED MYOGLOBINS.

## STUDIES ON ABSORPTION SPECTRA

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submitted in partial fulfillment of the requirements

for the degree Master of Science

in the Department of Chemistry

American University of Beirut

Beirut, Lebanon

June, 1968

AMERICAN UNIVERSITY OF BEIRUT

Thesis Title:

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Date of Thesis Presentation: 5/6/68.

## ABSTRACT

The $\epsilon$-amino groups of the lysine residues in sperm whale myoglobin were succinylated. The degree of modification was determined by reacting the modified protein with dinitrofluorobenzene and determining the amino acid composition. The succinylated myoglobin was characterized on a CM-Sephadex C-50 column.

The absorption spectra of sperm whale native, acetylated, and succinylated myoglobins and of horse native and acetylated myoglobins were measured at different pH values over the wavelength range of 650 - 200 mμ. The cyanide complex(es) of these myoglobins were also measured at different pH values over the same wavelength range. Comparison of the visible spectra of sperm whale acetylated and succinylated myoglobins and of horse acetylated myoglobin with the visible spectrum of the imidazole complex of sperm whale ferrimyoglobin suggested that the heme iron atom is not coordinated with a water molecule, as in the case of the native myoglobins, but with the imidazole nitrogen atom of the E 7 histidine. This would imply an increased flexibility in the tertiary structure, which is due to a loss in helical content of the myoglobin molecule. Further evidence for such an assumption was provided by the spectrophotometric titration of three tyrosine residues instead of two, as in native myoglobin.

An attempt to prepare a covalently cross-linked sperm whale myoglobin by use of the bifunctional reagent toluene-2,4-diisocyanate resulted in the formation of a high molecular weight polymer, as

shown by Sephadex G-75 chromatography. The ultraviolet spectrum of the polymeric sperm whale myoglobin indicated the attachment of a number of the aromatic residues of the reagent to the $\epsilon$-amino group of the lysine residues. The visible spectrum was similar to that of sperm whale succinylated myoglobin.

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| | |
|---|---|
| (n) Mb: | Native myoglobin |
| Ac-Mb: | Acetylated myoglobin |
| Suc-Mb: | Succinylated myoglobin |
| TC-Mb: | Covalently linked myoglobin |
| TC: | Toluene-2,4-diisocyanate |
| Mb IV: | Myoglobin component IV (Edmundson & Hirs, 1962) |
| Mb III: | Myoglobin component III (Edmundson & Hirs, 1962) |
| DNFB: | Dinitrofluorobenzene |
| DNP-: | Dinitrophenyl |
| BSA: | Bovine serum albumin |
| Cyt c: | Cytochrome c |
| $V_e$: | Elution volume |
| M: | Molecular weight |
| c: | Concentration |
| $\underline{M}$: | Molar |
| A: | Absorbance |
| $\epsilon$: | Molar absorbance |
| max.: | Maximum |
| min.: | Minimum |
| sh.: | Shoulder |
| isb.: | Isosbestic |

# INTRODUCTION

A protein molecule can be characterized by its amino acid composition and sequence, and by the configuration of its peptide chains in space. In the case of sperm whale myoglobin, Edmundson (1965) determined the complete amino acid sequence. Kendrew (1962) reviewed in detail the folding of the peptide chain in space, and the distribution and orientation of the side chains as they project from the peptide chain. The charged side chains, and the aliphatic hydroxyl groups of serine and threonine, are generally at the surface of the molecule, in contact with the solvent. These groups tend to be flexible and to move freely around their points of attachment to the main chain. The interior of the molecule is predominantly made up of non polar residues in van der Waals contact with one another. A few such residues, especially small ones like alanine, are at the surface.

The $\epsilon$-amino groups of lysine residues in sperm whale myoglobin, being found at the surface of the molecule can be modified specifically by treatment with organic reagents. In the course of studies of the chemical modification of protein antigens and their specific antibodies, Habeeb et al., (1958) have explored the reaction of succinic anhydride with bovine serum albumin, $\beta$-lactoglobulin and bovine $\gamma$-globulin. They observed large changes produced in the viscosity and the sedimentation behavior of these proteins upon modification, suggesting that a considerable expansion or unfolding

of the molecular structure had occurred. They also observed that bovine serum albumin and β-lactoglobulin were losing considerably their capacity to precipitate antibody specific to the respective unreacted proteins. From some further experiments, they concluded that the succinylated proteins lose their antigenic activity as a result of the attachment of the succinyl groups and not because of the reaction conditions. Maurer & Lebovitz (1956) reported on the reaction of gelatin with succinic anhydride. Although gelatin had been shown to be an efficient plasma volume expander, its use had been limited by its high gelation temperature. The modified fluid gelatin had a gelation temperature below 38°F. Terminiello et al., (1957) reported on the reaction of trypsin with succinic anhydride, but no relevant physiochemical studies accompanied these two last reports. An intensive study of the specificity of the reaction of bovine serum albumin with succinic anhydride and its conformational effects was made by Cherry (1964). Singer et al. (1966), reported that rabbit γ-globulin, succinylated either before or after partial reduction of disulfide bonds, could be separated into its constituent heavy and light peptide chains by gel filtration in aqueous buffers at pH near neutrality without the use of detergents. The lower retention of the succinylated chains by the gel as compared with the unmodified chains in 1M propionic acid may be due to an unfolding of the chains with a consequent enlargement of their molecular domains, or to aggregation. Moreover, the succinylated protein was more susceptible to reduction of its disulfide bonds than was the native protein. This observation was similar to that of Habeeb (1965), who

found that succinylation of bovine serum albumin increased the number of disulfide bonds susceptible to mild reduction, presumably as a result of the unfolding of the molecule produced by succinylation. Upon further studies on chemical modification of biologically active proteins, Habeeb (1966) presented a chemical method to assess the conformational changes associated with chemical modification of bovine serum albumin and human $\gamma$-globulin, based on the observation that conformational changes result in an increased reactivity of the disulfides towards $\beta$-mercaptoethanol, sodium sulfite and peracetic acid. Recently, Brunori et al. (1968) observed that acetylation and succinylation led to large changes in the pK' of the transition between the "acid" and the "alkaline" forms, which may be represented by the equation:

$$Fe(H_2O)^+ \rightleftharpoons Fe(OH) + H^+ \quad .$$

The ionization is generally attributed to the water molecule coordinated to the heme iron atom in the sixth position.

The cross-linking of two protein molecules has been reported by Moore & Ward (1956). They studied the cross-linking between bovine plasma albumin and wool keratin using N-substituted bis-maleimides. Sheehan & Hlavka (1957) reported the cross-linking of gelatin using a water soluble carbodiimide. They observed that on storing, the gelatin-carbodiimide mixture, the rate of disappearance of carboxyl groups paralleled the loss of free amino functions, evidence that amide bond formation was taking place. Singer (1959) prepared an electron dense antibody conjugate by covalently coupling ferritin to rabbit $\gamma$-globulin, containing a specific antibody. The purpose of

this work was to confer sufficient electron density upon the antibody molecule, without inactivating it, to render it visible in the electron microscope. Although, as mentioned above, a number of investigations had been made of the chemical cross-linking of protein molecules, the approach of Schick & Singer (1961) was different in several aspects. Schick & Singer were interested in preparing binary soluble conjugates between two different proteins and in retaining the biological activity of at least one of them. They, therefore, characterized the products of the coupling reactions more intensively than it had been done before. Conjugates of bovine serum albumin with bovine $\gamma$-globulin, as well as of ferritin with rabbit antibodies were prepared using diisocyanates. Diisocyanates were chosen as bifunctional coupling agents because they would react specifically with $NH_2$ groups on proteins; the reactivity was sufficiently great so as to function under mild conditions, yet was not so great as to decompse too rapidly in the reaction medium. They first used m-xylylene diisocyanate as a coupling agent because of the low reactivity of its primary aliphatic isocyanate groups; but they found that with m-xylylene diisocyanate-produced protein-protein conjugates, a substantial degree of non covalent linkage was involved. To make a conjugate which was exclusively covalently linked, they decided to employ a compound with two differentially reactive functional groups. The best results were achieved with toluene-2,4-diisocyanate (TC) because in this compound the para isocyanate group is considerably more reactive than the ortho. They found that the protein-protein conjugates prepared with TC were bound together through covalent, most probably ureido, linkages.

The present work is concerned with the use of succinic anhydride for the specific modification of the $\epsilon$-amino groups of lysine residues in sperm whale myoglobin. The extent of succinylation was determined by treating the chemically modified protein with dinitrofluorobenzene, which reacts with the free lysine groups. The protein was then hydrolyzed and its amino acid composition determined on the automatic analyzer. The modified myoglobin was further characterized by chromatography on a CM-Sephadex C-50 column. The preparation of covalently linked sperm whale myoglobin using toluene-2,4-diisocyanate is also described. The molecular weight of the resulting protein was determined by molecular sieve chromatography on Sephadex G-75. The absorption spectra of these two derivatives of sperm whale myoglobin, as well as the spectra of sperm whale acetylated myoglobin and horse acetylated myoglobin (S. Sabri, Thesis 1968), were measured and interpreted in the light of conformational changes due to steric factors and to altered electrostatic charge in the chemically modified derivatives.

## Amino Acid Analysis

In recent years, the amino acid sequence of a number of protein molecules (myoglobin, ribonuclease) have been deduced primarily from data obtained by the quantitative amino acid analysis of the peptides formed as a result of the action of trypsin, pepsin and chymotrypsin. Determinations of amino acids are also required in investigations of the distribution of amino acids and their derivatives in physiological fluids and mammalian tissues. Such studies were facilitated by the work of Moore, Stein, and Spackman on ion exchange chromatography and

photometric ninhydrin method which led to the construction of automatic equipment for continuous amino acid analysis. With an automatic analyzer, only 3 to 4 mg of protein sample and 24 hours are required for the quantitative evaluation of the amino acid composition of the protein.

The Technicon amino acid analyzer consists of two main parts: the chromatographic separation system and the analytical system. A schematic flow diagram of the auto-analyzer is shown in Figure 1 a and b.

The separation system is composed of a single heavy-walled, chromatographic column, 133 cm x 0.9 cm diameter, thermostated at $60^{0}$, and packed with a resin with spherical particles (Chromobeads). In their early work, Moore and Stein (1951) used for the separation of amino acid mixtures, columns packed with Dowex-50 resin with 8% cross-linking and they obtained very good separation. The chromatogram of amino acids on 16% cross-linked resins resulted in very poor separation. They concluded that the pore size of the resin was too small to permit rapid penetration of the amino acids into the resin particles and that the molecular weight had to be taken into account for the choice of the cross-linking of resins. But later, Moore and Stein (1954[a,b]) devised a procedure for the chromatographic determination of amino acids on 4% cross-linked sulfonated polystyrene resins. This kind of resin gave sharp peaks with small peptides as well as with amino acids. Using a buffer and an ionic strength gradient, all the amino acids could be separated on a 150 cm column, with the acidic amino acids eluting first and the basic amino acids last.

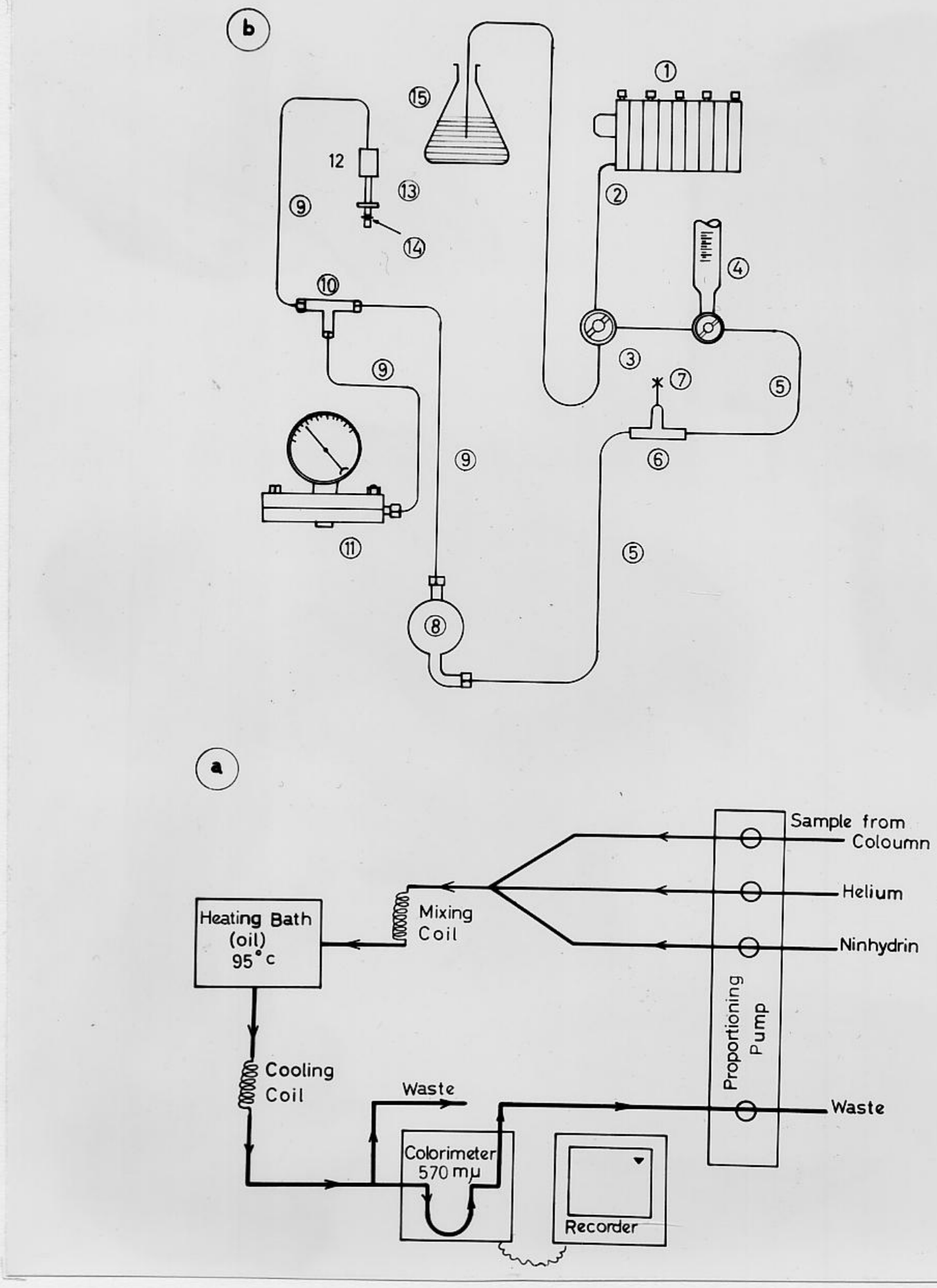


Figure 1 - Schematic flow diagram of Technicon amino acid analyzer (Technicon Manual).

(a) The analytical system

(b) The chromatographic separation system

1) Autograd
2) Teflon nipple
3) 3 way Teflon stopcock
4) 3 way Teflon stopcock and a flow gauge
5) Tygon sample tubing
6) de-bubbler 'T'
7) Screw clip
8) Milton Roy positive displacement pump
9) Teflon pressure tubing
10) 3 way Tylok 'T' piece
11) Pressure gauge
12) Tylok snubber
13) Column to line connector
14) Neoprene o-ring
15) Buffer reservoir

To render the analysis method precise and reproducible, the eluent is delivered at a constant and controllable rate by a positive displacement pump (Milton Roy). The pressure inside the column is usually of the order of 100 - 200 p.s.i.

The overall separation of the components in an amino acid sample is very strongly dependent on the pH of the eluent. Therefore, a convenient means is needed of making small changes in specific portions of the eluent gradient, as well as for presenting the gradient data in a manner to permit duplication. These requirements led to the development of a variable gradient mixer made up of 9 identical chambers connected together by valves: the Autograd (Peterson & Sober, 1959).

The analytical system of the auto-analyzer consists of a proportioning pump, a heating bath, a colorimeter and a recorder.

The proportioning pump consists of a pair of roller chains and a platen. As the rollers move, they contact the platen at various points. The effluent from the column, the ninhydrin reagent and the inert gas ($N_2$ or He) enter a manifold containing plastic tubes and placed between the roller and the platen. As the rollers move over the manifold, pressure occludes the tubes at the points of contact and this pushes the fluids forward. The volume of fluid aspirated and moved in the analytical system by this process is proportional to the inside diameter of the tubes. By selecting proper tube size and maintaining constant speed of operation, fixed proportion of sample, ninhydrin and inert gas are delivered to the system. This ensures precise and reproducible results.

The mixture of effluent, ninhydrin and gas from the manifold

flows through a coil of flexible tubing (95 ft.) immersed in an oil bath maintained at a constant temperature of 95°. Moore and Stein (1948, 1954b) studied the reaction between amino acids and ninhydrin reagent. They found that the reaction should be carried in an inert atmosphere, at 95° and in the presence of a strong reducing agent to eliminate the oxidative side reactions. They advised the use of hydrindantin as reducing agent.

The intensity of the blue color due to the reaction of amino acids with ninhydrin is determined by continuous photometry at 570 mμ. The colorimeter employs a single light source which provides twin beams. One light beam passes through the sample to a stable photocell; the other beam passes to an identical reference phdtocell. The ratio of the sample to the reference voltage is continuously measured in a null balance system in the recorder. The results are plotted continuously on the moving chart.

A more detailed presentation of the auto-analyzer can be found in the paper of Spackman et al., (1958).

## Visible and Ultraviolet Spectra of Proteins

Changes in absorption spectrum may often be among the first indications of structural change in a protein molecule. The use of ultraviolet spectra has undergone considerable refinement in the last few years especially, for the study of conformational changes in protein molecules.

The hemeproteins absorb light in the visible range of the

spectrum owing to the fact that iron-porphyrin complexes form part of their structure. The spectra of the hematin group in the visible and ultraviolet regions are intense and complicated, being derived in part from the characteristic absorption of the porphyrin conjugated double bond system and in part from the absorption of the iron atom. Theorell (1943) has suggested that the compounds may be devided into four classes, the division being based on the valence of the iron atom and on the kind of bonding in the two coordination positions of the iron atom which are not used by the iron-porphyrin bonds. The four classes are (I) $Fe^{+++}$ with ionic bonding, (II) $Fe^{+++}$ with covalent bonding, (III) $Fe^{++}$ with ionic bonding and (IV) $Fe^{++}$ with covalent bonding. All porphyrin compounds have also a very strong absorption band near 400 mμ. This band is called the Soret band.

The ultraviolet absorption spectra of proteins has been the subject of recent reviews by Beaven (1961), Wetlaufer (1962) and Edsall (1963). The simplest way of accounting for the absorption spectrum of a protein, in the ultraviolet region, is as the sum of the spectra of its components. This gives results which are often good approximations to the observed protein spectrum.

An examination of the spectra of the aromatic amino acids, phenylalanine, tyrosine and tryptophan shows that at wavelengths greater than 200 mμ, all three amino acids have spectra composed of two major absorption bands, a strong band at 210 - 220 mμ and a weaker band at 260 - 280 mμ (Malik, 1961). The separation of these two bands is approximately the same for all three aromatic chromophores. The spectrum of the phenolic-ionized tyrosyl group is similar to that of

un-ionized tyrosine, except that both its peaks are intensified and shifted about 20 mμ to longer wavelengths (Malik, 1961). This increase in absorptivity and the red shift in the spectrum accompanying ionization of the phenolic hydrogen provides the basis for studying the hydrogen ion equilibria of tyrosyl groups in proteins (Edsall & Wyman, 1958). Also, the displacement of the absorption peak in tyrosine from 275 to 295 mμ on titration with base, improves the resolution of tyrosyl from tryptophyl contributions in simultaneous spectrophotometric determination of these two residues. Certain modifications or derivatives of tyrosine have been also studied. Gemmill (1955) has reported the ultraviolet spectra of thyroxine. In the spectra of iodinated phenolic compounds, both the phenolic and the phenolate ion peaks are intensified and shifted to longer wavelengths. Spectral alterations of the tyrosyl spectrum resulting from the formation of an ether linkage with the phenolic oxygen are small, as seen in O-methyltyrosine (Wetlaufer et al., 1958). The absorption spectrum of phenylalanine shows a low intensity absorption peak centered at 257 mμ. But it is often obscured in proteins by the much stronger tyrosine and tryptophan absorptions. Tryptophan is the strongest absorber in the usual 270 - 290 mμ protein absorption band, its maximum absorptivity being about fourfold greater than un-ionized tyrosine and about twice as great as ionized tyrosine. The three aromatic amino acids exhibit absorption peaks in the range of 185 - 225 mμ.

The sulfur containing amino acids, cysteine, cystine, and methionine absorb in the region between 185 - 225 mμ. The ionization

of the thiol group of cysteine results in a strong increase in absorptivity, with a new peak forming in the wavelength region of 230 - 240 mμ (Benesch & Benesch, 1955). Methionine has a spectrum somewhat like that of cysteine, but its sulfur exhibits no acid-base behavior in the pH range of 1 to 13. The $-S-CH_3$, $-SH$ and $S-S-$ chromophores are especially sensitive to changes in environment and this may partly explain the frequent failure to obtain reproducible absorptivity values in protein spectra at the usual spectral minimum near 250 mμ.

The imidazole group of histidine absorbs appreciably in the region between 185 and 220 mμ. Because all three of the aromatic amino acids absorb strongly in this region, as well as cystine, methionine and cysteine, there have been no extensive studies on the absorption of histidyl residues in proteins. Unlike tyrosine or cysteine, ionization of the imidazole chromophore (ring nitrogen) perturbs the spectrum of histidine only very slightly (Saidel et al., 1952). The absorption spectrum of acetyl imidazole was reported by Stadtman (1954). Its absorption band is relatively broad, with $\lambda_{max} \simeq 244$ mμ and $\epsilon_{244} = 3.0 \times 10^3$.

Looking more closely to the absorption spectra of proteins, we can see that in the wavelength region between 250 and 300 mμ, the absorption is due almost entirely to the aromatic side chains of phenylalanine, tyrosine and tryptophan. The ionization of tyrosine residues in alkaline solutions can be conveniently followed by observation of the absorption band with its peak near 245 mμ. This is a characteristic of the ionized phenolic group and is several

times as intense as the absorption band centered at 295 mμ. In the case of sperm whale myoglobin, intensive studies have been done on the ionization of tyrosine groups in relation to the structure. Tyrosine residues have both polar and non-polar characteristics. The acidic phenolic group, with its capacity for hydrogen bonding, interacts readily with water. On the other hand, the large benzene ring gives the tyrosine side chain a strong tendency to imbed itself in non-polar regions of the molecule. Thus the tyrosine groups in myoglobin molecules and in proteins in general, may be found either in close contact with the solvent, or deeply buried in the interior of the molecule, or half buried, with the hydroxyl group near the surface, like the tryptophan groups of myoglobin which have the -N-H part of the ring at the surface and the large hydrophobic part of the ring buried inside the molecule. Hermans (1962) observed the ionization of the tyrosine groups in sperm-whale myoglobin and in human and horse hemoglobin, by measuring the absorbance changes at 245 mμ. In hemeproteins measurement at 245 mμ also has the advantage of involving less overlap with the characteristic absorption bands of the heme group. Hermans found that of the three tyrosines in myoglobin two groups ionize reversibly, one with a pK value of 10.3 and the other with a pK value of 11.5. A third group was apparently quite unavailable for ionization in the native molecule. It appears natural to identify the latter group with the tyrosine residue at H 22 which Kendrew (1962) finds to be hydrogen bonded to a CO group of the peptide chain in the interior of the molecule. Breslow (1962) has shown that, if the heme group of myoglobin is removed, in the

resulting globin molecule all three tyrosine residues are titrable and the entire spectrophotometric titration curve is shifted to lower pH values. Moreover there exists a profound difference between the tyrosine groups of insulin and those of ovalbumin. In insulin all four of the tyrosine residues are readily and reversibly ionizable with an intrinsic pK of 9.60 $\pm$ 0.1 (Tanford & Epstein, 1954) whereas the nine or ten tyrosine groups of ovalbumin are virtually unavailable for reaction with the solvent medium until the molecule is denatured in strong alkaline solution above pH 12 (Crammer & Neuberger, 1943). Therefore we can see that the situation is very complex and even when the tyrosines are apparently free to ionize reversibly, as in serum albumin, they are clearly very different from tyrosine groups in simple peptides (Tanford et al., 1955a, b). The instrinsic ionization constant of the albumin tyrosine groups, corrected for electrostatic interactions, is characterized by the unusually high pK value of 10.4. The "normal" intrinsic pK values of appropriate small molecules is 9.6 $\pm$ 0.2 and values in this range have been calculated for poly-L-tyrosine ($pK_{int}$ = 9.5) and insulin ($pK_{int}$ = 9.6) (Tanford & Epstein, 1954). The heat of ionization is 11.5 kcal/mole, in contrast to the normal value of about 6, and $\Delta S^{o}$ is -9 e.u. as against the normal value near -25 e.u. for tyrosine groups. There have been two general hypotheses regarding the nature of the forces that prevent ionization of tyrosine groups in native protein molecules. On the one hand, as was first suggested by Crammer and Neuberger (1943), the tyrosine hydroxyl groups may be hydrogen bonded to suitable acceptor groups, for

instance to the ionized carboxyl groups of aspartate or glutamate residues, or, as in the one actually established case in myoglobin, to a C = O group in the main peptide chain. On the other hand the tyrosyl groups may be shielded from the solvent by enclosure in a surrounding sheath of non-polar side chains, in the native protein molecule (Yanari & Bovey, 1960). But these two proposed mechanisms, should not be regarded as mutually exclusive. It is highly likely that the "unavailable" tyrosine groups in proteins are hydrogen bonded and also buried in the predominantly non-polar interior of the molecule.

Extensive studies on simple peptides showed that they all have a high intensity absorption band in the neighborhood of 190 mμ. Proteins show a similar band in the same region. Moreover, N-acetyl and N-glycyl derivatives of almost all common amino acids show the same band at 190 mμ. These facts taken together constitute strong evidence for associating the 190 mμ band with the peptide bond in proteins. Imahori and Tanaka (1959) were the first to report a marked change in the ultraviolet absorption of polypeptides near 190 mμ, accompanying the helix-coil transition. The absorption of the random coil, in the region near 190 mμ, was found to be markedly greater than that of the helix. Recent work of Rosenhech and Doty (1961) has extended these observations to other polypeptides and to certain proteins. If the attempt is made to draw conclusions concerning the percentage of helix in a protein from ultraviolet absorption at about 190 mμ, a number of calculations are necessary. A knowledge of the amino acid composition is also required, with

appropriate corrections for the contribution of the various side chains, amide groups and ionized carboxyl groups to the absorption at the given wavelength.

# EXPERIMENTAL

## Materials

Myoglobin. Sperm whale (Skeletal muscle) salt free, lyophilized was purchased from Severac Laboratory (Maidenhead, Berks, England) and was used without further purification.

Acetylated myoglobin. Sperm whale and horse were prepared by Mr. Salim Sabri (Thesis, 1968).

Bovine serum albumin, fraction IV, was purchased from Sigma Chemical Company (St. Louis 18, Missouri).

Cytochrome c was purchased from Nutritional Biochemicals Corporation (Cleveland, Ohio).

Toluene-2,4-diisocyanate. (K & K Laboratories, Inc. Plainview, New York) was used without further treatment.

Ninhydrin was purchased from Pierce Chemical Company (Rockford, 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 proceed for 30 min. without further heating. During the next hour the solution was cooled to room temperature under running tap water. The hydrindantin was then filtered off, washed well with water and dried in a vacuum desiccator over $P_2O_5$. The yield was 95%.

Ethyl cellosolve (Oxitol) was obtained 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 at $4^{\circ}$.

Polyoxyethylene (23) Lauryl ether (Brij 35 SP), (Atlas Chemical Industries, Wilmington, Delaware). The solution of Brij was prepared 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/gram and the particle size was 40 - 120 $\mu$.

Sephadex G-75, bead polymerized, fine grain (Pharmacia, Uppsala, Sweden). As quoted by the manufacturers, the water regain was $7.5 \pm 0.5$ grams of water imbibed per gram dry gel, the particle size was 40 - 120 $\mu$ and the bed volume per gram dry gel was 12 - 15 ml.

Blue Dextran 2000, was purchased from Pharmacia, Uppsala, Sweden.

1,10-Phenanthrolin, perchloric acid (70%) and pyridine were products of Merck (Darmstadt, Germany) of 'pro analysi' grade.

Hydroxylamine hydrochloride (Fisher Scientific Company) had a maximum specified impurity of 1 ppm Fe.

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

#### Succinylation procedure

Sperm whale and horse Mb were succinylated according to the procedure of Habeeb *et al.*, (1958). To 60 ml of a 5% solution of Mb in water, adjusted to pH 8.0 with 1N NaOH, 3 g of succinic anhydride (12 moles per mole of free amino groups) were added with continual magnetic stirring keeping the temperature of the reaction mixture at $0^{\circ}$. The pH was maintained at 8.0 by adding 1N NaOH with a micro-syringe. After half an hour reaction, the solution was dialyzed exhaustively against water at $4^{\circ}$ and the product was lyophilized. The modified Mb was stored in a tightly closed bottle at $4^{\circ}$. The yields were: sperm whale Suc-Mb 3.13 g; horse Suc-Mb 1.87 g.

#### Conjugation procedure

The procedure given by Schick & Singer (1961) was followed. To 100 ml of a 1.5% sperm whale Mb solution in phosphate buffer pH 7.5, I = 0.1, 2 ml of toluene-2,4-diisocyanate (TC) were added at $0^{\circ}$. After vigorous stirring in an ice bath for 25 min., the solution was centrifuged at $0^{\circ}$. The supernatant was removed and was allowed to stand for one hour at $0^{\circ}$.

The above solution was added to an equal volume of 1.5% sperm whale Mb solution in borate buffer pH 9.5, I = 0.1 at $37^{\circ}$. After one hour of vigorous stirring at $37^{\circ}$, the reaction mixture was

dialyzed exhaustively against water containing a few crystals of $(NH_4)_2\ CO_3$ and the product was lyophilized.

## Amino acid analysis

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

Some minor modifications were introduced: helium gas was used instead of nitrogen, since the required purity of nitrogen was difficult to obtain; 80 ml of buffer were used to fill each of the autograd chambers instead of 75 ml; 0.1% phenol was added in the citrate buffers for protection against bacteria (van Stekelenburg, 1966).

The following solutions were prepared for an amino acid run:

Sodium acetate buffer, 4.0N pH 5.5. Anhydrous sodium acetate (820 g) were added slowly to approximately 1 liter of water with constant magnetic stirring. The solution was cooled to room temperature and 250 ml of glacial acetic acid were added slowly. Then water was added up to 2500 ml.

Ninhydrin. The ninhydrin was prepared directly in a 10 liter dark bottle connected to the proportioning pump and to the helium tank. Helium gas, was bubbled continuously inside the bottle during the preparation. Oxitol 1300 ml, was put in the dark vessel and after 15 min. stirring, 40 g of ninhydrin and 3 g of hydrindantin were added to the Oxitol. After another 15 min. of stirring, 700 ml of sodium acetate buffer (4.0N, pH 5.5) and 6000 ml of a 1:1 mixture of water and Oxitol were added. Helium was bubbled in the solution for at least 30 min. before use.

Citrate buffer pH 2.875. $Na_3C_6H_5O_7.2\frac{1}{2}\ H_2O$ (16.85 g) or $Na_3C_6H_5O_7$

(14.71 g) were dissolved in 900 ml of water. To this solution, 25.0 ml of 2.00N NaOH, 5.0 ml of thiodiglycol, 10.0 ml of Brij 35 solution and 1 g phenol were added. The mixture was titrated with 6N HCl to a pH of 2.875 using a Radiometer Type PHM 4 pH-meter. The volume was brought to 1000 ml by adding water and the pH was adjusted again to 2.875 if necessary.

Citrate buffer pH 3.80, was prepared exactly the same way as the citrate buffer, pH 2.875. 6N HCl was added so as to bring the pH down to 3.80.

Citrate buffer pH 5.00. $Na_3C_6H_5O_7.2\frac{1}{2}\ H_2O$ (16.85 g) or $Na_3C_6H_5O_7$ (14.71 g) were dissolved in 900 ml of water. 25.0 ml of 2.00N NaOH, 35.07 g NaCl, 10 ml Brij 35 and 1 g phenol were added to the solution. The pH was adjusted to 5.00 by adding 6N HCl. Water was added to a total volume of 1000 ml and the pH was checked again.

Carbonate buffer pH 10.0. The buffer was prepared by mixing 275 ml of 0.2M $Na_2CO_3$ and 225 ml 0.2M $NaHCO_3$. To this solution 1.5 g of phenol and water up to a total volume of 1500 ml were added.

Standard glycine solution (1mM), was prepared by dissolving 7.51 mg of glycine in 0.1N HCl, the final volume being 100 ml.

Standard amino acid mixture (1mM). The solution was prepared by putting the desired amounts of amino acids in a 250 ml volumetric flask and, adding 50 ml of methanol, and diluting to volume with 0.1N HCl. The mixture was 1mM with respect to the following 18 amino acids: Arg, nor-Leu, Asp, Thr, Ser, Glu, Pro, Gly, Ala, Cys, Val, Met, Ile, Leu, Tyr, Phe, Lys, His .

Preparation of dinitrophenyl (DNP) derivative of Mb. The

method given by Wofsy & Singer (1963) was followed. Modified or native Mb (50 mg) in 1 ml water was denatured by adding 2 ml ethanol. After one hour, 0.1 ml dinitrofluorobenzene (DNFB) and 0.2 mg $NaHCO_3$ were added. The mixture was put on a shaker for at least 2 hours at room temperature. The insoluble protein was removed by centrifugation and was washed twice with water, twice with ethanol, and twice with ether.

Myoglobin and DNP-myoglobin hydrolysates were prepared according to the method of Moore & Stein (1963), with some modifications. The protein (5 mg) was placed in a heavy-walled pyrex tube and 1 ml of 4N HCl was added. The mixture was frozen in liquid air and sealed under vacuum (0.025 mm Hg) using an oxygen flame. The sealed tube was then left at $110^{\circ} \pm 1$ for 24 hours. After cooling, the tube was opened and the hydrolysate was lyophilized and stored at $4^{\circ}$. In the case of the hydrolysis of native Mb, 1 ml of 6N HCl was used.

Amino acid analyzer. A glass chromatographic column, 140 cm x 0.64 cm diam., thermostated at $60^{\circ}$ was used. The flow rate was regulated with a Milton Roy mini pump to 0.5 ml/min. at a pressure between 100 and 200 p.s.i. The column was packed with Chromobeads type A gel and was eluted with citrate buffer pH 2.875 or with a buffer gradient, from the autograd, during a run. The autograd was filled as described in Table I. For the elution of DNP-lysine, 80 ml of carbonate buffer was added to the autograd 19 - 20 hours after the beginning of the run. At the end of the run, the column was washed with 0.2N NaOH for half an hour followed by elution with citrate buffer pH 2.875 for at least 6 hours before the beginning of

Table I

Buffer Gradient

| Chamber | 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 |

the following run.

To standardize the system, a solution was made containing 2.5 ml of 1mM amino acid mixture, 6 ml of DNP-lysine (1mM) and 1.5 ml of a 1:1 mixture of 0.1N HCl and 40% sucrose. A 0.5 ml portion of this solution was applied to the amino acid analyzer column using an Agla micrometer syringe. The final concentration in the standard mixture was 0.125mM with respect to each amino acid. A myoglobin hydrolysate sample for analysis was prepared in a similar way by dissolving the hydrolysate in 5 ml of a 1:1 mixture of 0.1N HCl and 40% sucrose. Of this solution 0.5 ml were applied to the column. After the preparation of a new batch of ninhydrin, a standard amino acid mixture was applied to the column and the ninhydrin color values for each amino acid were obtained from the peaks on the chart of the amino acid analyzer. The results of two calibration runs are shown in Table II. The color equivalent of ninhydrin was checked before every run, using a standard glycine solution. The areas under the peaks were calculated by multiplying half the maximum value of the absorbance by the width of the peak at half the maximum absorbance*.

## Column chromatography

### CM-Sephadex C-50 chromatography

A glass chromatographic column (Pharmacia, Uppsala, Sweden) 25 cm x 1.27 cm diam., was packed with CM-Sephadex C-50 in 0.05M phosphate buffer pH 6.85, which was also used as an eluent. The sample was prepared in the same buffer. The density of the solution was increased by adding solid sucrose to a concentration of 15 - 20%. All solutions were filtered before being applied to the column.

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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 31 the error will cancel out, since relative values are calculated.

Table II

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 | \|ΔArea\| | % 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 | 0.00 |
| 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 | 0.00 |

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

The sample, 0.50 ml, was applied to the column using an Agla micrometer syringe (Burroughs Wellcome & Co, London, England). A time-operated fraction collector (Gilson Medical Electronics, Middleton, Wisconsin) was used. The chromatographic runs were done in the cold room at 4°. The volume of each fraction was measured by weight difference between the empty test tube and the filled test tube. All samples were analyzed by reading absorbancies at 410 mμ and 280 mμ on either a Zeiss PMQII spectrophotometer or a Hitachi Perkin-Elmer UV-VIS 139 spectrophotometer.

Sephadex G-75 chromatography

A glass chromatographic column, 69 cm x 1.27 cm diam., was packed with G-75 Sephadex in 0.05M phosphate buffer pH 7.02. The flow rate was 4.2 ml/hour. The same procedure was followed as described above for the CM-Sephadex C-50 column. The G-75 column was calibrated by running samples of cytochrome c, sperm whale (n) myoglobin and bovine serum albumin (see Results section).

## Spectra

Stock solutions of native myoglobin and myoglobin derivatives (10 ml solutions of 50 mg protein/ml) were prepared for sperm whale (n) Mb, sperm whale Ac-Mb, spermwhale Suc-Mb, horse (n) Mb and horse Ac-Mb. The concentrations of the stock solutions were determined by iron analysis.

The following buffers were used:

Acetate buffers. Stock solutions of 0.2M acetic acid (11.55 ml $CH_3COOH$/1000 ml) and 0.2M sodium acetate (16.4 g of $NaC_2H_3O_2$/1000 ml)

were prepared. The acetate buffers in the range of pH 3.6 - 5.6, of constant molarity, 0.05M in acetate, were obtained by mixing different proportions of the two stock solutions. The final pH of each buffer solution was read on a Radiometer pH meter to ± 0.005.

Carbonate buffers. The following stock solutions were prepared: 0.2M sodium carbonate (21.2 g $Na_2CO_3$/1000 ml) and 0.2M sodium bicarbonate (16.8 g $NaHCO_3$/1000 ml). The carbonate buffers in the pH range of 9.2 - 10.7, of constant molarity, 0.05M in carbonate, were obtained by mixing different proportions of the two stock solutions. The final pH of each buffer solution was read on a Radiometer pH meter to ± 0.005.

Citrate buffers. The stock solutions prepared were the following: citric acid, 0.1M (21.01 g of $C_6H_8O_7.H_2O$/1000 ml) and sodium citrate, 0.1M (33.4 g of $Na_3C_6H_5O_7.5\frac{1}{2}H_2O$/1000 ml). The citrate buffers, in the pH range of 3.0 - 6.2, of constant molarity, 0.05M in citrate, were obtained by mixing different proportions of the two stock solutions. The final pH of each buffer solution was read on a Radiometer pH meter to ± 0.005.

Glycine buffers. The following stock solutions were prepared: glycine, 0.2M (15.01 g $NH_2CH_2COOH$/1000 ml) and 0.2M NaOH. The glycine buffers, in the pH range of 8.6 - 10.6, of constant molarity, 0.005M in glycine, were obtained by mixing different proportions of the two stock solutions. The final pH of each buffer solution was read on a Radiometer pH meter to ± 0.005.

Phosphate buffers. Stock solutions of sodium dihydrogen orthophosphate, 0.2M (31.20 g $NaH_2PO_4.2H_2O$/1000 ml) and di-sodium hydrogen

orthophosphate, 0.2M (28.39 g $Na_2HPO_4$/1000 ml) were prepared. The phosphate buffers, in the range of pH 5.7 - 8.0, of constant molarity, 0.05M in phsophate, were obtained by mixing different proportions of the two stock solutions. The final pH of each buffer was read on a Radiometer pH meter to ± 0.005.

Phosphate, borax buffers. A stock solution, 0.01M in disodium hydrogen phosphate (1.42 g $Na_2HPO_4$/1000 ml) and 0.01M in borax (3.81 g $Na_2B_4O_7.10H_2O$/1000 ml) was prepared. To the needed volume of buffer, enough 2N NaOH was added to bring the pH above 12 and then was titrated on a Radiometer pH meter to the desired pH using 6.0N HCl.

Buffers containing cyanide. Acetate buffer (0.05M, 0.1% KCN), glycine buffer (0.05M, 0.1% KCN), phosphate buffer (0.05M, 0.1% KCN), phosphate borax buffer (0.1% KCN) were prepared by disolving solid KCN in the buffers and reading the pH on a Radiometer pH meter to ± 0.005.

## Iron determination

The procedure given by Cameron (1965) was followed.

To a 10 ml volumetric flask, 0.2 ml of myoglobin solution (50 mg/ml), 0.2 ml of 30% $H_2O_2$ and 0.2 ml 70% $HClO_4$ were added. The mixture was digested on a steam bath for half an hour. After cooling, 0.1 ml of 10% hydroxylamine hydrochloride solution, 1 ml of 1% 1,10-phenanthrolin solution (in 50% ethanol) and 2 ml of pyridine were added. The solution was diluted to volume with water and the absorbance at 510 mμ was read on a Hitachi Perkin-Elmer UV-VIS 139 spectrophotometer. The blank absorbance values were usually in the range of 0.025 to 0.030. A solution of $Fe^{++}$, previously standardized

by titration against $K_2Cr_2O_7$ was used to verify Beer's law (Fig. 2). All the glassware was previously soaked in concentrated nitric acid and rinsed with doubly distilled water.

## Spectral Measurements

All spectra were taken on a Perkin-Elmer 450 UV-Visible-NIR spectrophotometer.

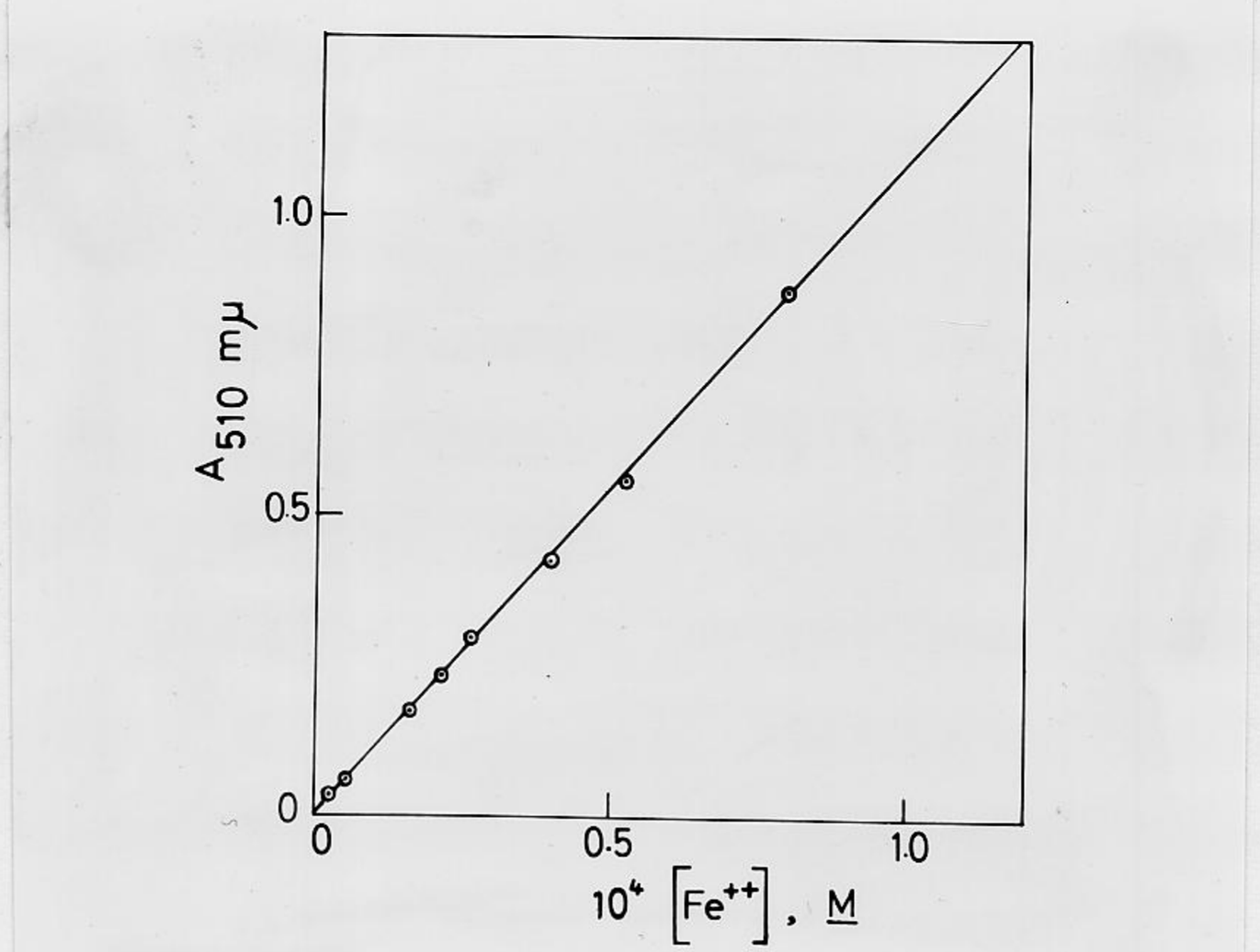



Figure 2 - Validity of Beer's law in the iron determination.

## 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,

$S_{A_{rg}}$ is the area of the Arg peak in the standard amino acid mixture,

$S_{aa}$ is the area of the particular amino acid in the standard amino acid mixture run,

$A_{A_{rg}}$ is the area of Arg peak in the myoglobin hydrolysate,

$A_{aa}$ is the area of the particular amino acid in the hydrolysate,

$N_{Arg}$ is the literature value for the number of Arg residues in myoglobin and is equal to 4 in sperm whale Mb and to 2 in horse Mb.

Arginine was chosen as internal standard, because of the consistent results obtained between runs. Table III shows that the values obtained for amino acid residues were consistent with literature values.

The extent of modification of Suc-Mb was obtained by treating the modified myoglobin with DNFB and then hydrolysing the resulting

Table III

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)

derivative with 4N HCl for 24 hours under vacuum and 110°. The succinyl lysines were hydrolyzed, but the DNP-lysines were resistent under the conditions stated.

The hydrolysates were analyzed for their amino acid content and the results are recorded in Table IV. It can be seen that the number of lysine residues has changed from 19 to 17.3, that is to say there has been 91% succinylation of sperm whale myoglobin. The values for all the other amino acids have remained constant. In the case of horse myoglobin, there was no appreciable extent of succinylation, since the number of lysine residues dropped from 19 to 0.9, showing that there was only 4.6% succinylation of the lysine residues in horse myoglobin.

CM-Sephadex C-50 chromatography. Sperm whale (n) Mb and sperm whale Suc-Mb were applied to the C-50 Sephadex column separately. The elution volume was 85.5 ml in the case of sperm whale (n) Mb IV and 10.5 ml in the case of sperm whale Suc-Mb (Fig. 3a and b). The succinylated derivative is seen to contain no appreciable amount of unmodified myoglobin. A mixture (4:1) of sperm whale Suc-Mb and sperm whale (n) Mb was also applied to the column and a very good separation was obtained (Fig. 3c). The elution volumes were identical with the ones obtained when each component was chromatogramed separately.

Sephadex G-75 chromatography. The Sephadex G-75 column was calibrated by running samples of Blue Dextran, bovine serum albumin, cytochrome c, sperm whale (n) Mb and $K_2CrO_4$. The elution profiles were determined at the wavelengths listed in Table V. The $V_e$ values obtained (Table VI) were plotted versus the logarithm of the molecular weights for each of these substances, as shown in Fig. 4. The elution

Table IV

Number of Lysine Residues Obtained by Amino Acid Analysis of DNP-Modified Mb.

| Species | Chem. Mod. | No. of Lys in native Mb | No. of Lys modified | % Lys modified |
|---|---|---|---|---|
| Sperm whale Mb | Suc | 19 | 17.3 | 91 |
| Horse Mb | Suc | 19 | 0.9 | 4.6 |

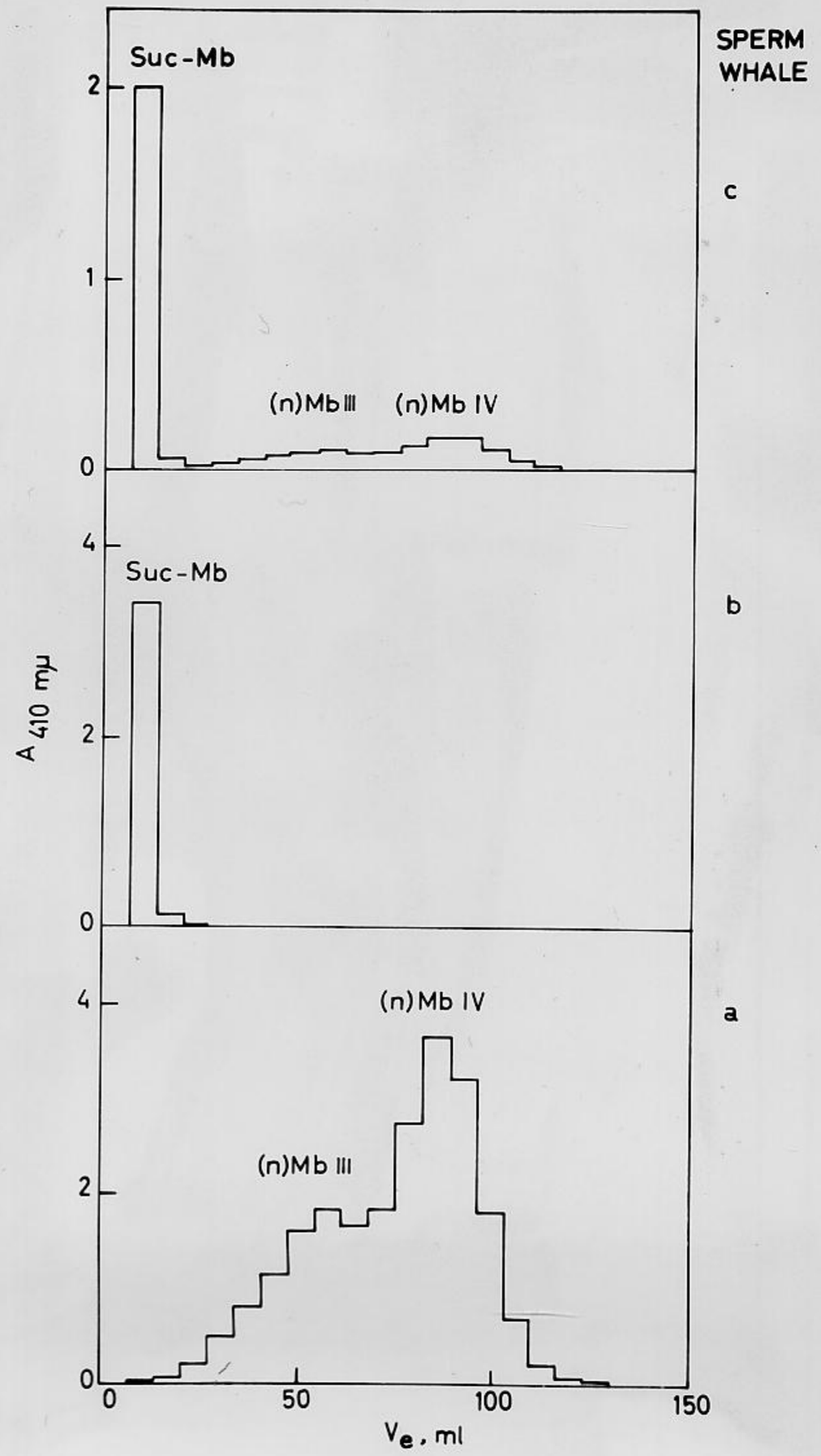



Figure 3 - Elution profile of sperm whale (n) Mb and sperm whale Suc-Mb on CM-Sephadex C-50 column, 0.05M phosphate buffer, pH 6.85.

(a) Elution profile of sperm whale (n) Mb
(b) Elution profile of sperm whale Suc-Mb
(c) Elution profile of a 1:4 mixture of sperm whale (n) Mb and sperm whale Suc-Mb

Table V

Solutions Used for the Calibration

of the Sephadex G-75 Column

| Sample | M | Conc. applied to column | $\lambda$, mμ |
|---|---|---|---|
| Blue Dextra | $2 \times 10^6$ (Pharmacia Lit.) | 2.5 mg/ml | 262 |
| BSA | 67,000 (Loeb & Scheraga, 1956) | 30 mg/ml | 280 |
| Sperm whale (n) Mb | 17,816 (Edmundson, 1965) | 30 mg/ml | 410 |
| | | | 280 |
| Cyt c, monomer | 12,384 (Margoliash, 1962) | 30 mg/ml | 414 |
| $K_2CrO_4$ | 194 | 0.4M | 460 |

Table VI

Molecular Weight Determination from Elution Volume on Sephadex G-75 Column.

| Sample | $V_e$, ml | M | Log M |
|---|---|---|---|
| Blue Dextran | 30.4 | $2 \times 10^6$ | 6.30 |
| BSA | 33.8 | 67,000 | 4.83 |
| Sperm whale (n) Mb | 59.8 | 17,816 | 4.25 |
| Cyt c, monomer | 63.8 | 12,384 | 4.09 |
| $K_2CrO_4$ | 99.6 | 194 | 2.29 |
| Sperm whale TC-Mb | 29.0 | (>70,000) | (4.97) |
| | 61.7 | 15,000 | 4.18 |

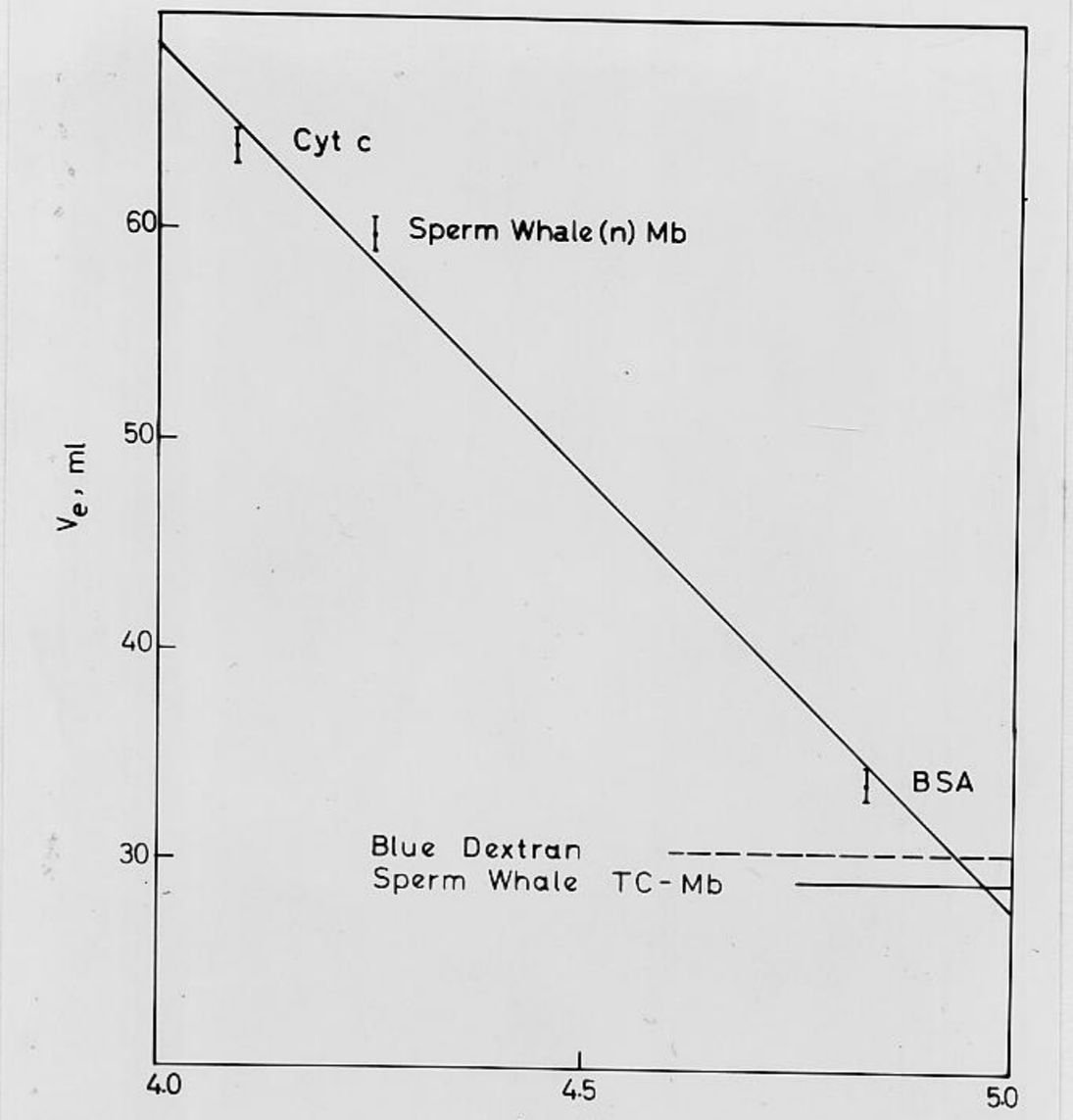



Figure 4 - Dependence of Sephadex G-75 elution volumes on molecular weight.

0.05M phosphate buffer pH 7.02

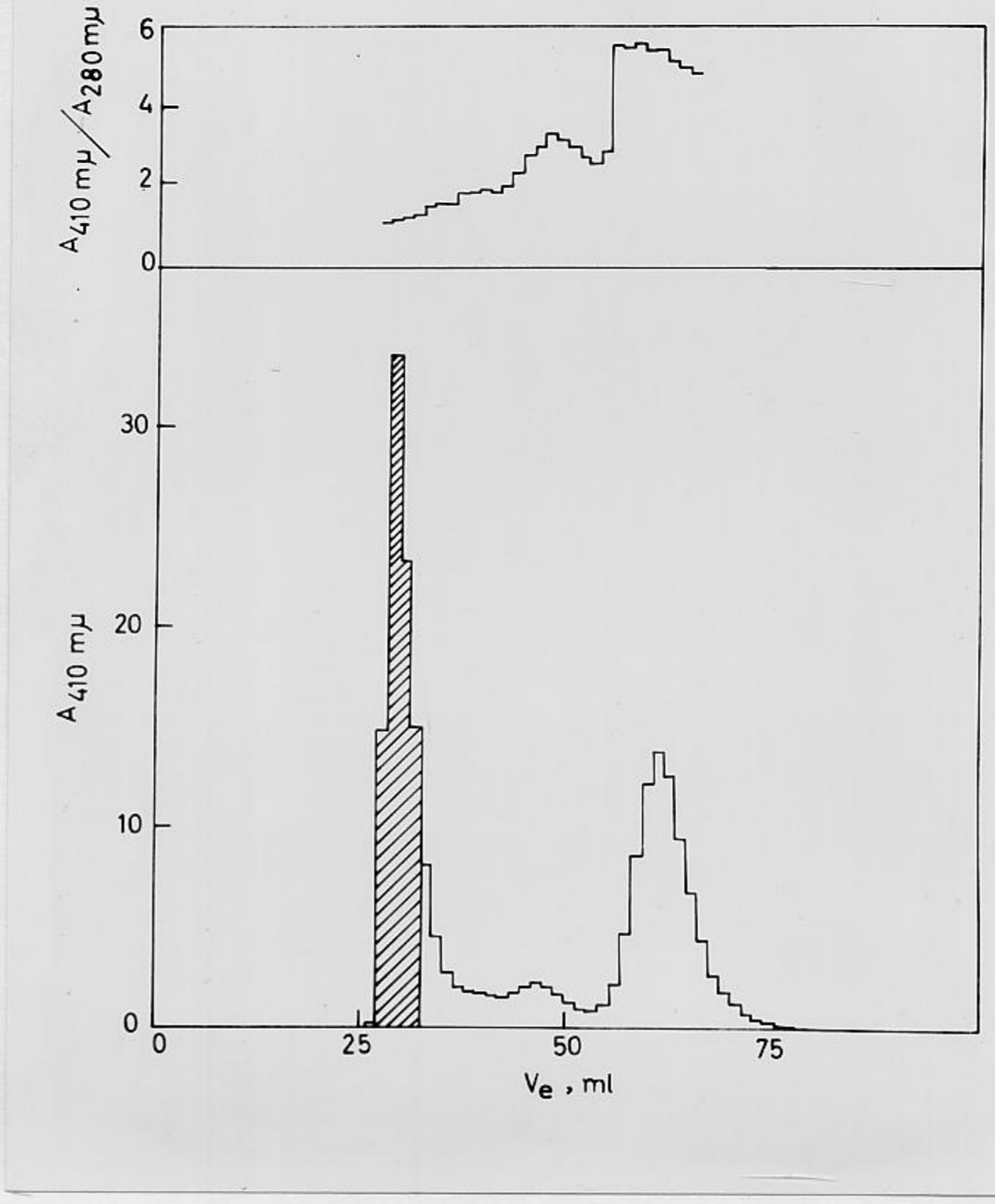



Figure 5 - Elution profile of sperm whale TC-Mb on Sephadex G-75.

0.05M phosphate buffer pH 7.02

volume was found to be linearly dependent on log M in the range of molecular weight of 10,000 - 70,000, in agreement with the work of Andrews (1962) and Whitaker (1963).

A sample of the covalently linked myoglobin (TC-Mb), 80 mg in 2 ml of 0.05M glycine buffer, pH 9.37 containing 0.3 g of sucrose was filtered and chromatographed on a Sephadex G-75 column. The final volume of the solution applied to the column was 1.8 ml. The elution profile obtained was composed of three peaks as seen in Fig. 5. The second and third peaks were identified as being native sperm whale Mb, because the elution volumes were 46.6 ml and 61.7 ml respectively as compared to 44.7 and 59.8 ml obtained in the case of the chromatogram of sperm whale (n) Mb. Moreover, the ratio of the 410 mμ/280 mμ absorbance was approximately 3.2 for the second peak and approximately 5.4 for the third peak, the latter as expected for sperm whale (n) Mb. A cut was taken of the effluent containing sperm whale TC-Mb, between the elution volumes of 27.0 and 32.4 ml (shaded area in Fig. 5). The ratio of the 410 mμ/280 mμ absorbance for this portion had a value near 1.2. This solution of sperm whale TC-Mb, in 0.05M phosphate buffer, pH 7.0, was stored at $4^{\circ}$. The concentration of TC-Mb, determined by iron analysis, was $2.14 \times 10^{-4}$M. The visible and ultraviolet spectra of this solution were measured.

Spectra. The absorption spectra of native and modified sperm whale and horse myoglobins were measured in the visible and ultraviolet regions at different pH's. The spectra of the cyanide complex with native and chemically modified myoglobin were also studied with variations in pH.

(a) The absorption spectra of sperm whale (n) Mb, sperm whale Ac-Mb, sperm whale Suc-Mb, horse (n) Mb and horse Ac-Mb, in 0.05M phosphate buffer, pH 7.05 were measured. The values of the absorbances at characteristic wavelengths, as well as the corresponding $\epsilon$ values, are listed in Tables VII - XI (see Appendix). These values were plotted against wavelength (Figs. 6 - 11). It can be seen that in the visible region the peaks at 630 and 580 mμ which are present in the native sperm whale and horse myoglobins have completely disappeared in the modified proteins. Moreover, the absorption maximum at 502 mμ in the native myoglobins has shifted to higher wavelength by 31 mμ in the case of sperm whale Ac and Suc-Mb and by 32 mμ in the case of horse Ac-Mb. The Soret band has also undergone a red shift of 4.5 mμ in sperm whale Ac-Mb and horse Ac-Mb and of 3 mμ in sperm whale Suc-Mb, concomitant with a marked fall in absorption intensity for all modified proteins. In the ultraviolet region, a shoulder appears at 365 mμ in the absorption spectra of sperm whale Ac-Mb, sperm whale Suc-Mb, and at 360 mμ in the spectra of horse Ac-Mb. This shoulder does not exist in the parent native myoglobins. The absorption peak of native sperm whale and horse myoglobins with a maximum at 280 mμ disappears in the ultraviolet spectra of sperm whale Ac-Mb and horse Ac-Mb; but it can still be seen in sperm whale Suc-Mb, with a fall in absorption intensity and a blue shift of 8 mμ.

(b) Variation of the absorption spectra of sperm whale Ac-Mb with pH was studied. The absorption spectra were measured in the pH range of 8.62 - 10.20 and 9.45 - 12.02 and the detailed characteristics of these curves are given in Table XII (see Appendix). Isosbestic points

at 578 mμ, 500 mμ, 485 mμ, 392 mμ were observed indicating clearly the presence of two distinct species.

(c) Fig. 12a and b show the absorption spectra of sperm whale (n) Mb, sperm whale Ac-Mb, sperm whale Suc-Mb, horse (n) Mb and horse Ac-Mb at pH 2.69 and 5.65 between 750 mμ and 450 mμ. The sperm whale (n) Mb at pH 5.65 exhibits the characteristic spectrum of acidic ferrimyoglobin with the two small peaks at 630 and 580 mμ and the high intensity absorption peak at 502 mμ. At pH 2.69, the peak at 630 mμ has shifted to 632 mμ, the peak at 580 mμ as well as the peak at 500 have almost completely disappeared. In the case of sperm whale and horse Ac-Mb, at pH 5.65, the spectra exhibit the maximum absorption at 533 mμ; but at pH 2.69 this peak cannot be seen any more. In the case of sperm whale Suc-Mb, at pH 5.65 the derivative does not already have the peak at 533 mμ, it has a very broad band with a maximum at 523 mμ; the protein precipitates at a pH of 2.69.

Fig. 13 shows the absorption spectra of sperm whale (n) Mb, sperm whale Ac-Mb, sperm whale Suc-Mb at pH 9.45 and 12.02 between 750 mμ and 450 mμ, and 450 - 350 mμ. Sperm whale (n) Mb, at pH 9.45 and 12.02 has very well defined peaks in the visible region with maxima at 580 mμ, 540 mμ and 410 mμ and 414 mμ (at pH 12.02). Sperm whale Ac-Mb in pH 9.45 has well defined peaks with maxima at 533 and 412 mμ; but in a buffer of pH 12.02, the peak at 533 is almost completely lost, as well as the Soret peak which is very broad and with a maximum shifted to 390 mμ. In the case of sperm whale Suc-Mb, at pH 9.45 and 12.02, the peak at 533 mμ and the Soret band have disappeared.

(d) Variations in the absorption spectra,with pH, of the cyanide

complex of sperm whale (n) Mb, sperm whale Ac-Mb, sperm whale Suc-Mb, hore (n) Mb and horse Ac-Mb were studied in the entire visible and ultraviolet regions. Three wavelengths were chosen to follow the influence of pH (pH 5.6 - 12.0) on the absorption of the derivatives. The wavelengths of 360 and 540 mμ were selected, because they are characteristic of the heme group, and the wavelength of 245 mμ was chosen, because the ionization of tyrosine residues in alkaline solutions can be conveniently followed at that wavelength (Edsall, 1963). The different values of $\epsilon$ corresponding to different pH values are listed in Tables XIII - XV (see Appendix). Fig.14 represents the plots of $\epsilon$ against pH at the different wavelengths of 245, 360 and 540 mμ. It can be seen that the $\epsilon$ values at 540 mμ and 300 mμ of sperm whale (n) MbCN and horse (n) MbCN remain almost constant. In contrast, the $\epsilon$ values of sperm whale Ac-MbCN, sperm whale Suc-MbCN and horse Ac-MbCN vary with pH. At 245 mμ, the ionization of the tyrosine residues can be seen at pH above 9.5.

(e) Absorption spectra of sperm whale TC-Mb, in 0.05M phosphate buffer pH 7.05 were measured. The values of the absorbances at characteristic wavelengths as well as the corresponding $\epsilon$ values are listed in Table XVI (see Appendix). These $\epsilon$ values are plotted against wavelength (Fig. 15). It can be seen that in the visible region, the absorption maximum at 502 mμ in sperm whale (n) Mb, has shifted to 525 mμ. The Soret band has a maximum at 410 mμ instead of 408 mμ in the native myoglobin. The peak with a maximum at 280 mμ, in the ultraviolet spectrum of sperm whale (n) Mb, is not seen in the spectrum of sperm whale TC-Mb; after 300 mμ, the absorption intensity of TC-Mb increases continuously.

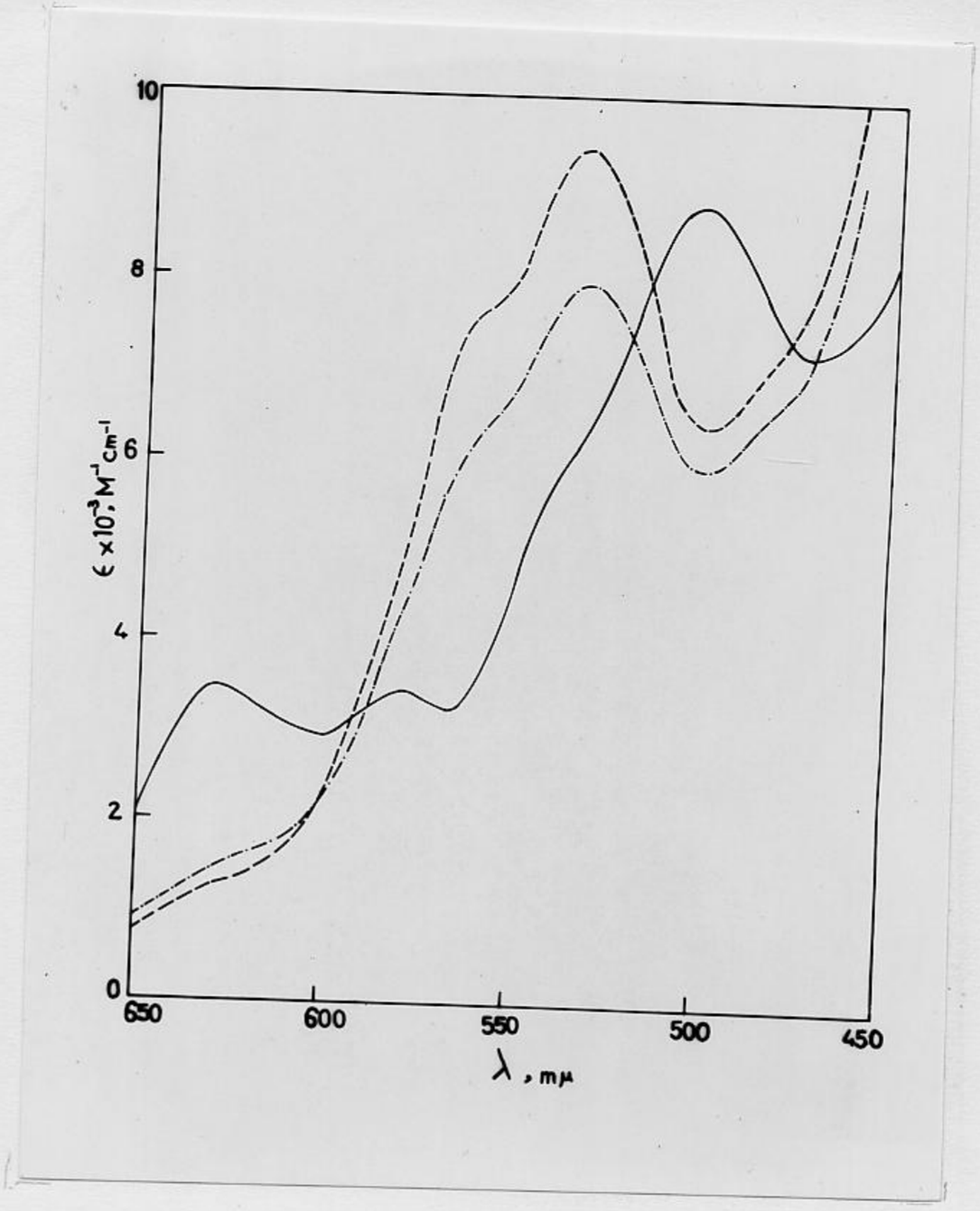



Figure 6 - Absorption spectra of sperm whale (n) Mb (_____), sperm whale Ac-Mb ( - - - - - ), sperm whale Suc-Mb ( -.-.-. ) in 0.05M phosphate buffer pH 7.05: 650 - 450 mμ.

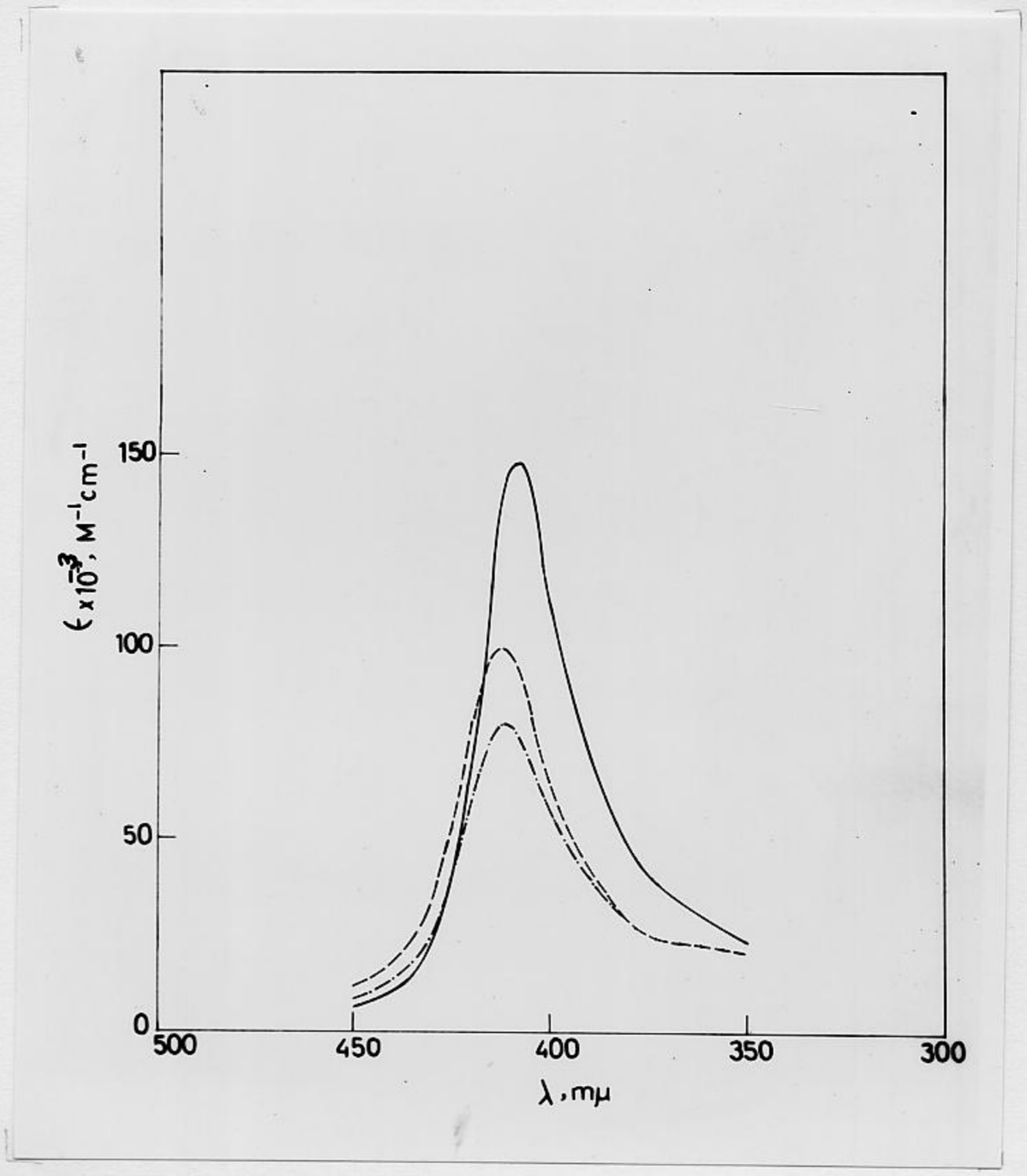



Figure 7 - Absorption spectra of sperm whale (n) Mb (————),
sperm whale Ac-Mb (-----), sperm whale Suc-Mb ( -.-.-. )
in 0.05M phosphate buffer pH 7.05: 450 - 350 mμ.

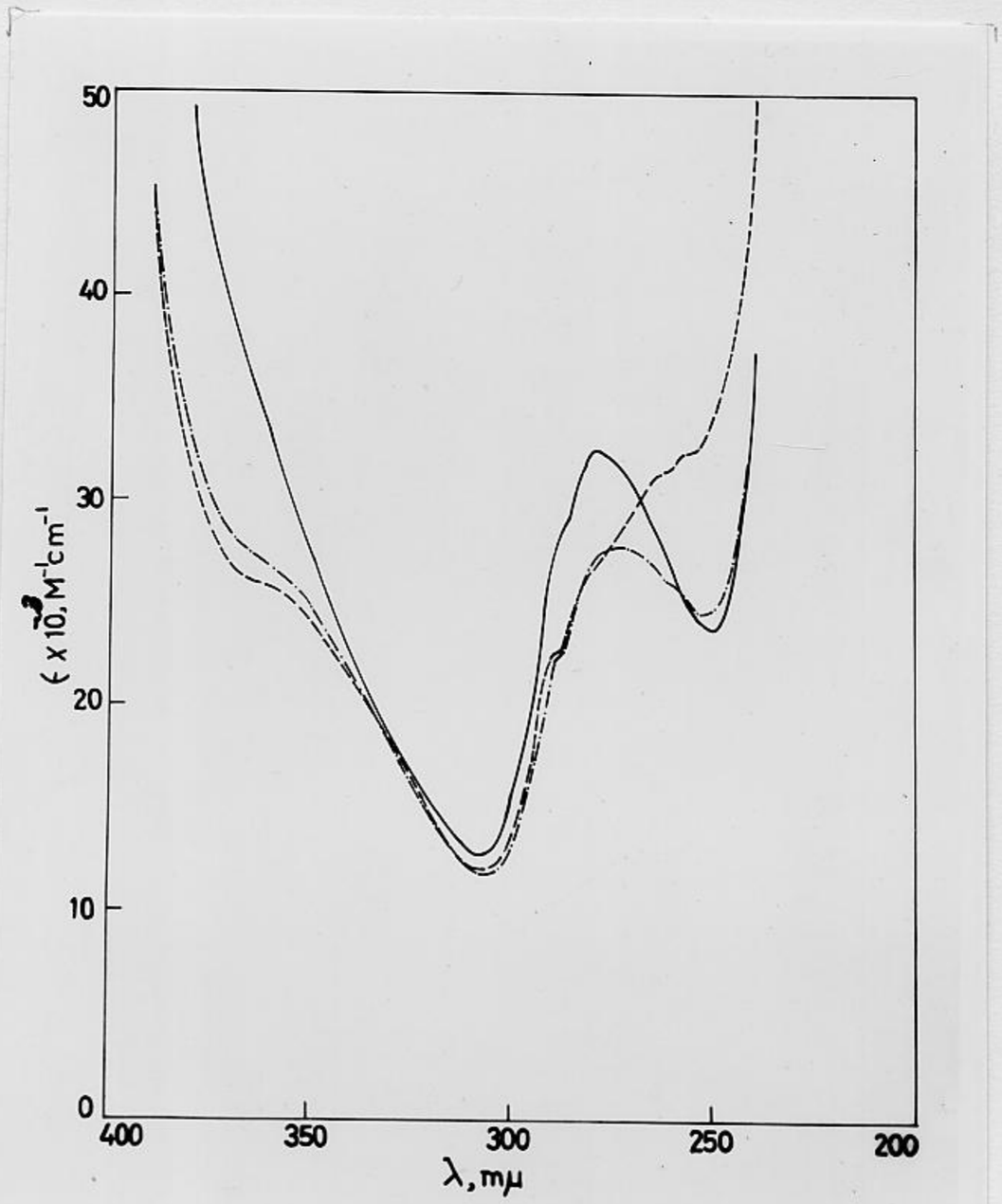



Figure 8 - Absorption spectra of sperm whale (n) Mb (———), sperm whale Ac-Mb ( ----- ), sperm whale Suc-Mb (-.-.-.) in 0.05M phosphate buffer pH 7.05: 400 - 200 mμ.

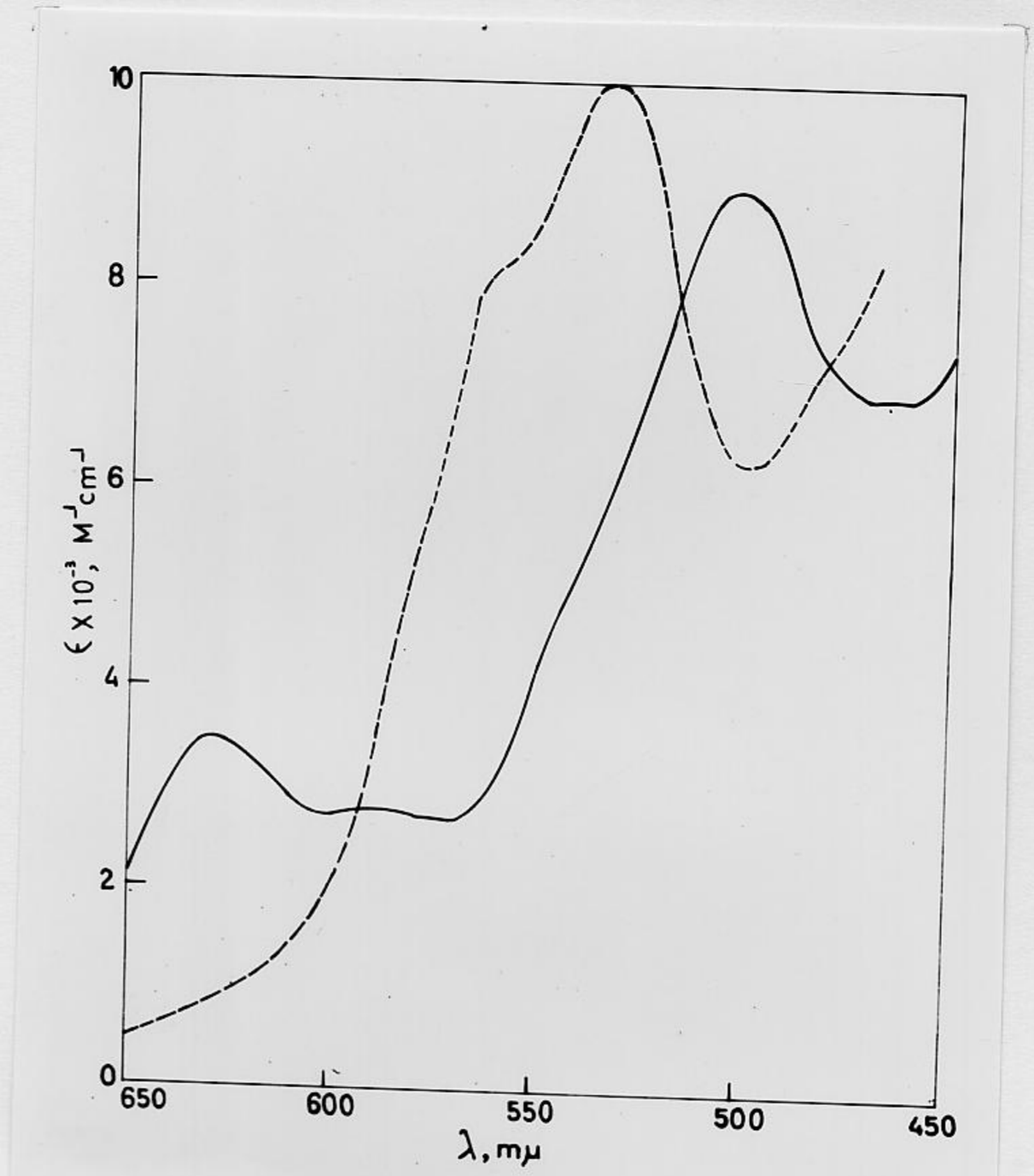



Figure 9 - Absorption spectra of horse (n) Mb (_____), horse Ac-Mb (- - - - -) in 0.05M phosphate buffer pH 7.05: 650 - 450 mμ.

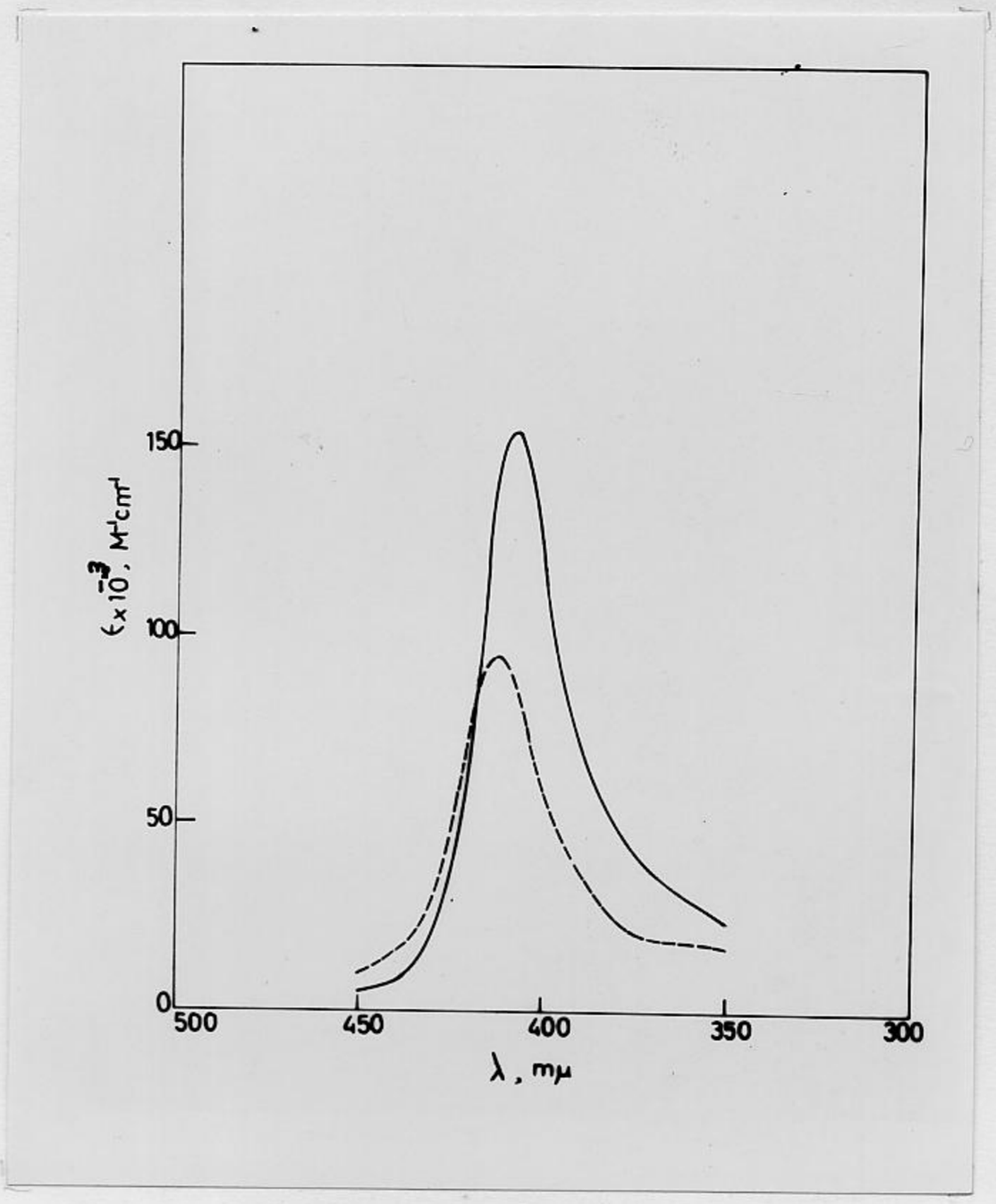



Figure 10 - Absorption spectra of horse (n) Mb (——), horse Ac-Mb ( ----- ), in 0.05M phosphate buffer pH 7.05: 450 - 350 mμ.

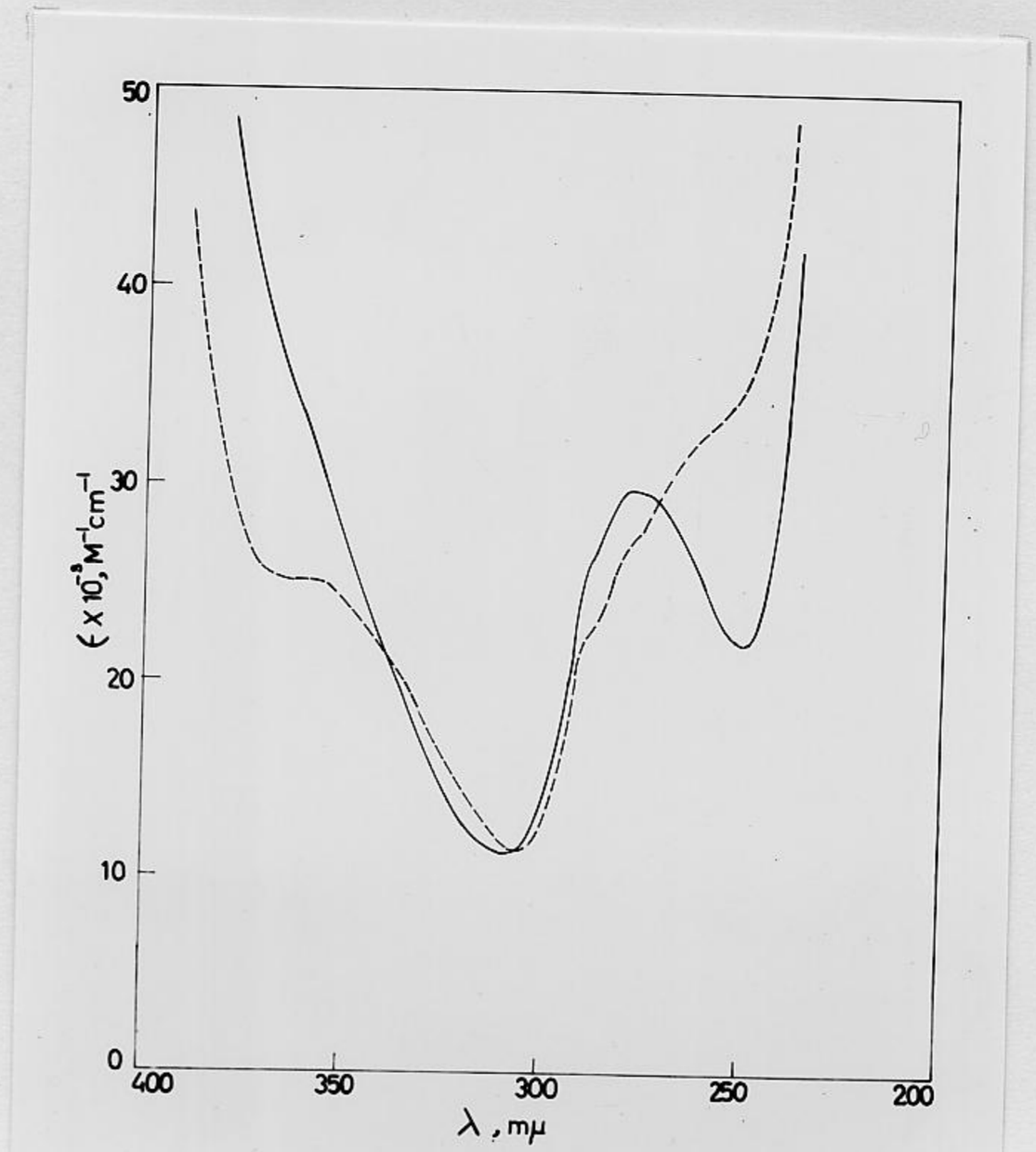



Figure 11 - Absorption spectra of horse (n) Mb (———), horse Ac-Mb (- - - - -) in 0.05M phosphate buffer pH 7.05: 400 - 200 mμ.

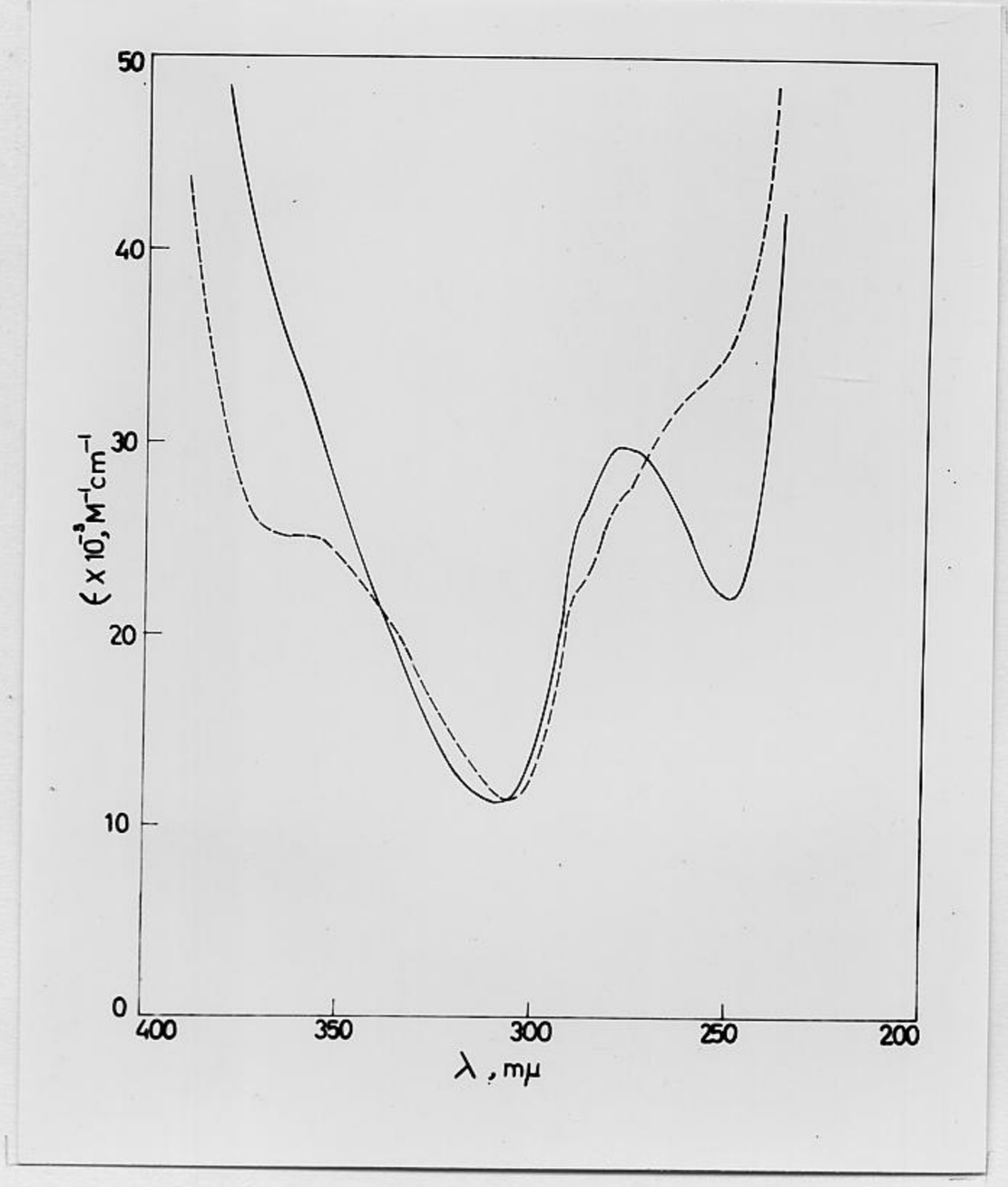



Figure 11 - Absorption spectra of horse (n) Mb (———), horse Ac-Mb (-----) in 0.05M phosphate buffer pH 7.05: 400 - 200 mμ.

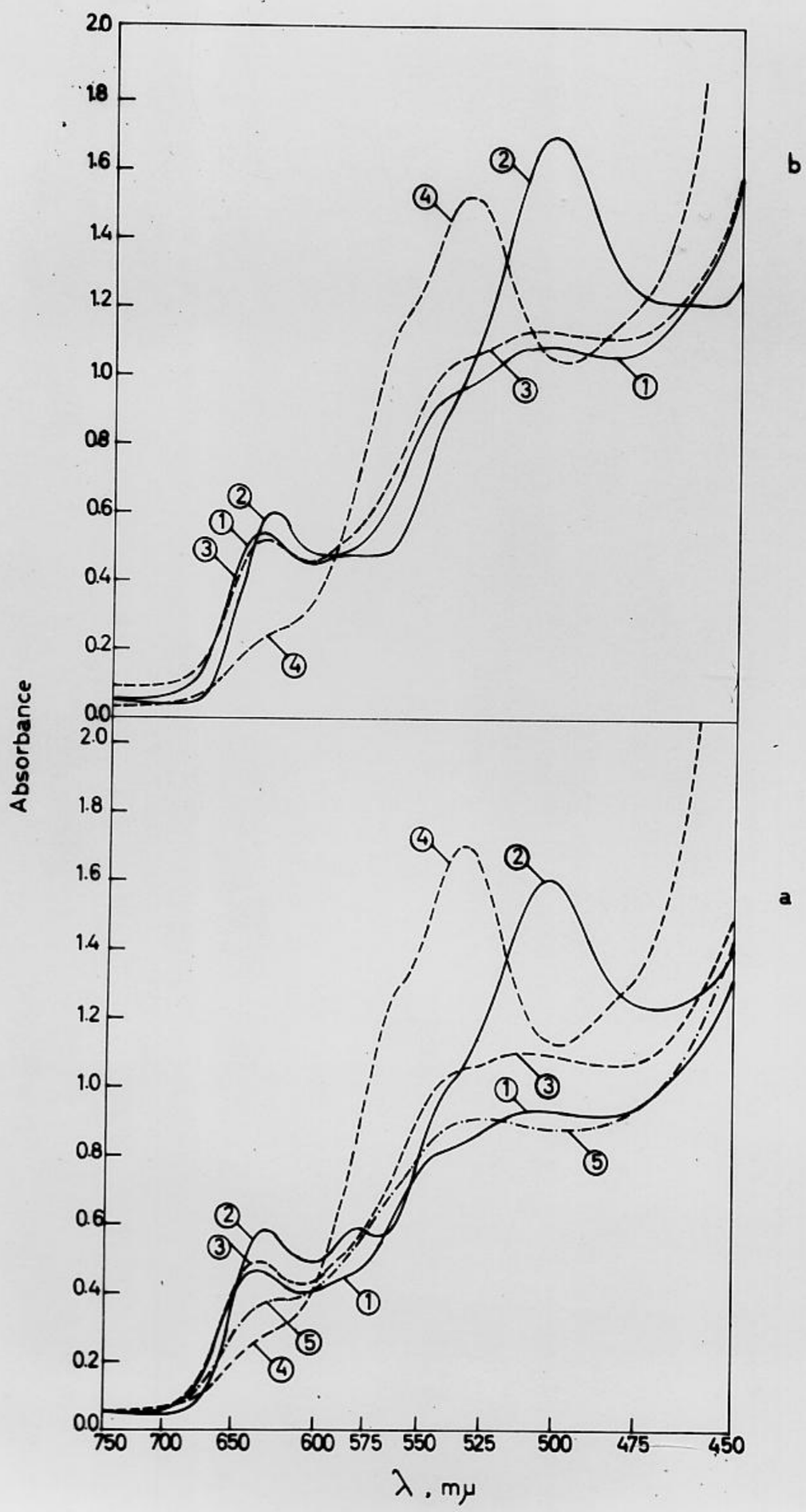



Figure 12 - Absorption spectra (pH 2.69 and 5.65)

(a) (1) ——— Sperm whale (n) Mb ($1.4 \times 10^{-4}$ M) in 0.05M citrate buffer pH 2.69
(3) ----- Sperm whale Ac-Mb ($1.5 \times 10^{-4}$ M) in 0.05M citrate buffer pH 2.69
(2) ——— Sperm whale (n) Mb ($1.4 \times 10^{-4}$ M) in 0.05M acetate buffer pH 5.65
(4) ----- Sperm whale Ac-Mb ($1.5 \times 10^{-4}$ M) in 0.05M acetate buffer pH 5.65
(5) -.-.-.Sperm whale Suc-Mb ($1.3 \times 10^{-4}$ M) in 0.05M acetate buffer pH 5.65

(b) (1) ——— Horse (n) Mb ($1.9 \times 10^{-4}$ M) in 0.05M citrate buffer pH 2.69
(3) ----- Horse Ac-Mb ($1.8 \times 10^{-4}$ M) in 0.05M citrate buffer pH 2.69
(2) ——— Horse (n) Mb ($1.9 \times 10^{-4}$ M) in 0.05M acetate buffer pH 5.65
(4) ----- Horse Ac-Mb ($1.8 \times 10^{-4}$ M) in 0.05M acetate buffer pH 5.65

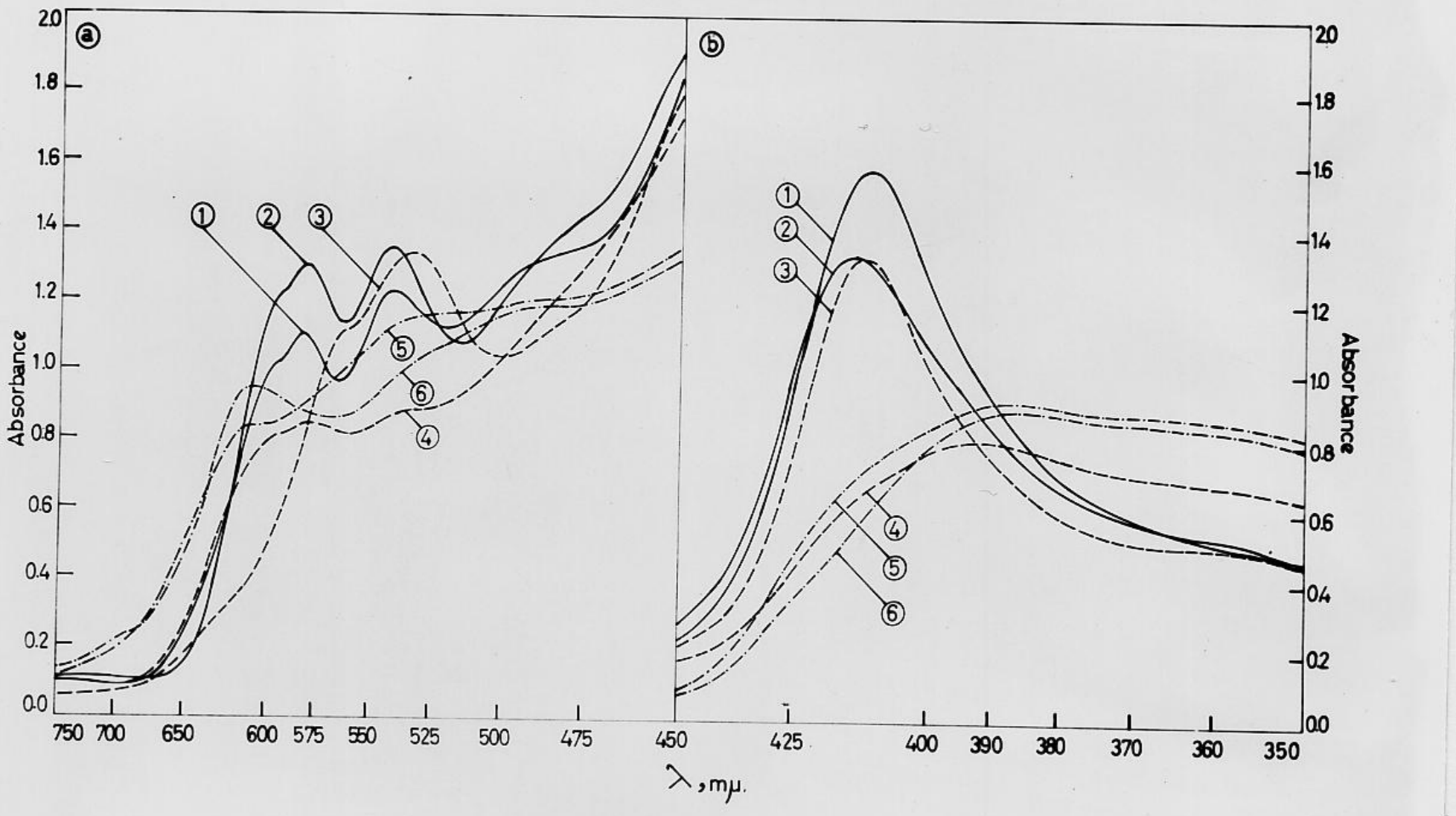



Figure 13 - Absorption spectra (pH 9.45 and 12.02)

(a) (1) ——— Sperm whale (n) Mb ($1.4 \times 10^{-4}$ M) in phosphate borax buffer pH 9.45
(3) ----- Sperm whale Ac-Mb ($1.5 \times 10^{-4}$ M) in phosphate borax buffer pH 9.45
(5) -.-.-. Sperm whale Suc-Mb ($1.3 \times 10^{-4}$ M) in phosphate borax buffer pH 9.45
(2) ——— Sperm whale (n) Mb ($1.4 \times 10^{-4}$ M) in phosphate borax buffer pH 12.02
(4) ----- Sperm whale Ac-Mb ($1.5 \times 10^{-4}$ M) in phosphate borax buffer pH 12.02
(6) -.-.-. Sperm whale Suc-Mb ($1.3 \times 10^{-4}$ M) in phosphate borax buffer pH 12.02

(b) (1) ——— Sperm whale (n) Mb ($1.4 \times 10^{-5}$ M) in phosphate borax buffer pH 9.45
(3) ----- Sperm whale Ac-Mb ($1.5 \times 10^{-5}$ M) in phosphate borax buffer pH 9.45
(5) -.-.-. Sperm whale Suc-Mb ($1.3 \times 10^{-5}$ M) in phosphate borax buffer pH 9.45
(2) ——— Sperm whale (n) Mb ($1.4 \times 10^{-5}$ M) in phosphate borax buffer pH 12.02
(4) ----- Sperm whale Ac-Mb ($1.5 \times 10^{-5}$ M) in phosphate borax buffer pH 12.02
(6) -.-.-. Sperm whale Suc-Mb ($1.3 \times 10^{-5}$ M) in phosphate borax buffer pH 12.02

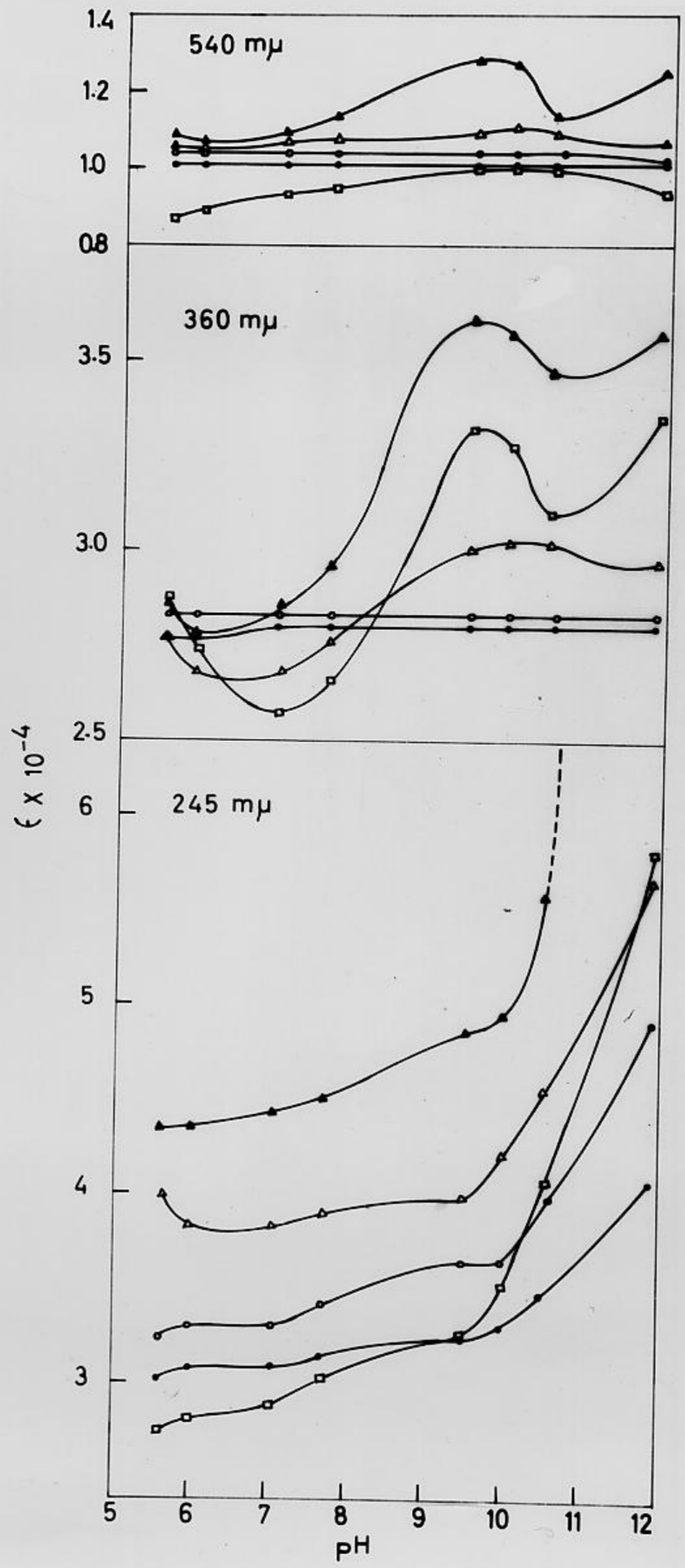



Figure 14 - pH-Profile of molar absorbances at $\lambda$ = 245, 360 and 540 mμ.

○ Sperm whale (n) MbCN

△ Sperm whale Ac-MbCN

□ Sperm whale Suc-MbCN

● Horse (n) MbCN

▲ Horse Ac-MbCN

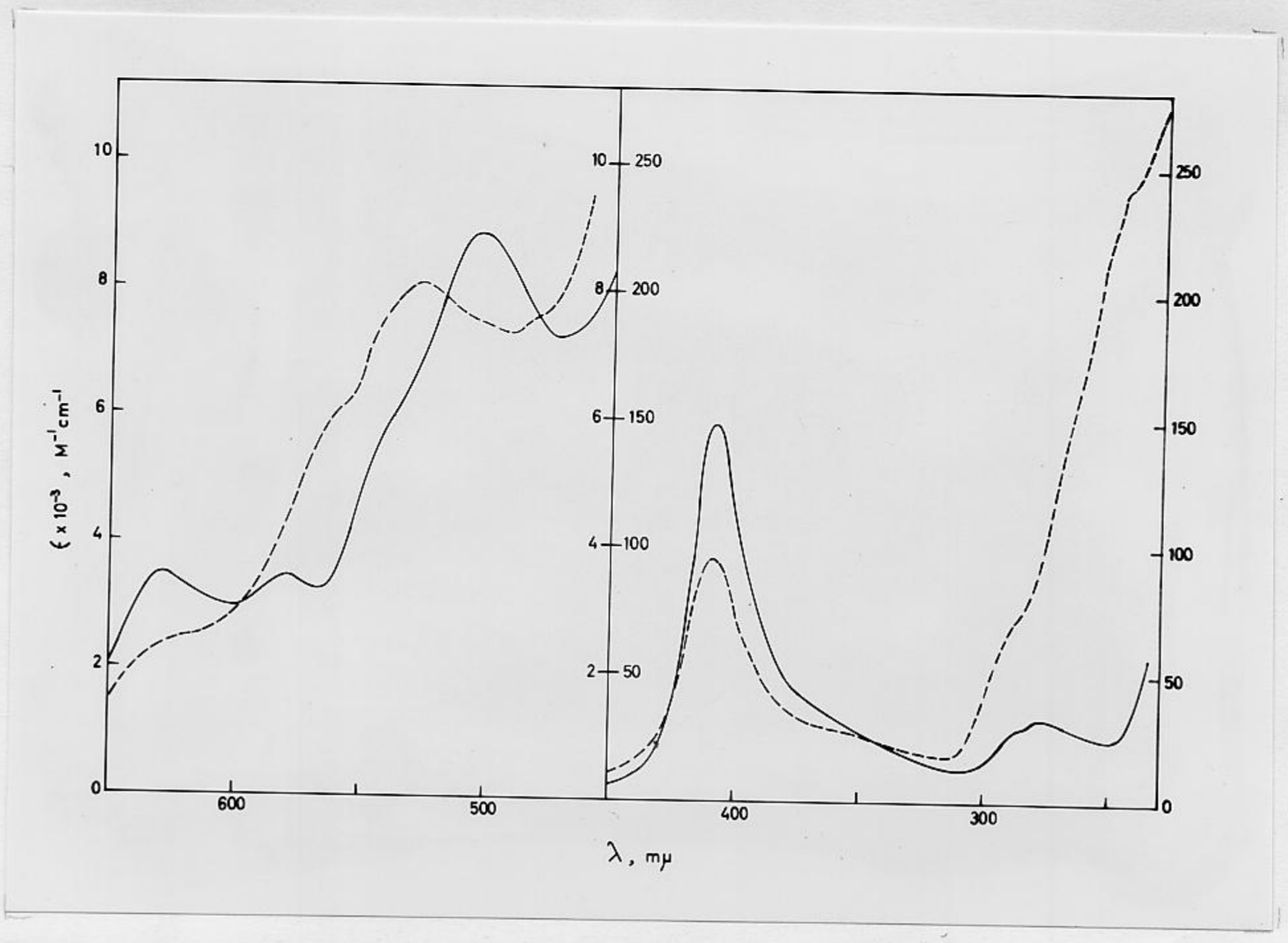



Figure 15 - Absorption spectra of sperm whale (n) Mb (____) and sperm whale TC-Mb (-----) in 0.05M phosphate buffer pH 7.05.

## DISCUSSION

The reaction of the lysine residues in sperm whale (n) Mb with succinic anhydride resulted in the replacement of the positively charged $\epsilon\text{-}NH_3^+$ groups by the negatively charged $-NHCOCH_2CH_2COO^-$ groups. This difference in charge between the native and the modified myoglobins explains the lower retention of the succinylated derivative on CM-Sephadex cation exchanger as compared to the native myoglobin. In the case of sperm whale Ac-Mb and horse Ac-Mb, the positively charged $\epsilon\text{-}NH_3^+$ groups of lysines have been replaced by the neutral group $-NHCOCH_3$ (Sabri, Thesis, 1968).

The visible spectra of sperm whale Ac-Mb, sperm whale Suc-Mb and horse Ac-Mb at pH 7.05 show great similarities with the visible spectrum of the imidazole complex of sperm whale (n) Mb. The imidazole complex of sperm whale (n) Mb has a single absorption band in the visible (534 mμ) and a shoulder at 560 mμ (Baghdoyan, Thesis 1965). Similar bands have been observed with other nitrogen ligands, such as azide (Havemann, 1952) and pyridine (Barrow, 1937). A comparison of the spectral characteristics of sperm whale acidic ferrimyoglobin, sperm whale alkaline ferrimyoglobin, cyanide complex of ferrimyoglobin, imidazole complex of ferrimyoglobin, sperm whale Ac-Mb and sperm whale Suc-Mb is given in Table XVII. These data provide strong evidence that, in the modified myoglobins, the sixth coordination position of the heme iron atom is no longer occupied by a water molecule; instead the iron atom is directly coordinated to the imidazole group of the

Table XVII

Characteristics of absorption spectra of sperm whale acidic ferrimyoglobin, alkaline ferrimyoglobin, cyanide complex of ferrimyoglobin, imidazole complex of ferrimyoglobin, Ac-Mb and Suc-Mb.

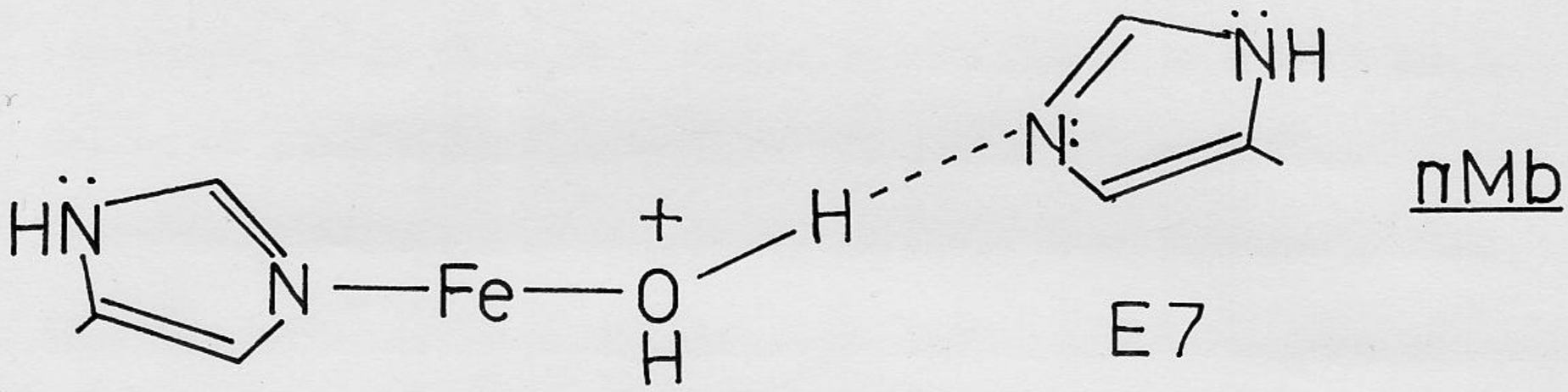
| $Fe^{+}(H_2O)$ | $Fe(OH)^{(1)}$ | $Fe-CN^{(1)}$ | $Fe-ImH^{(2)}$ | Ac-Mb | Suc-Mb |
|---|---|---|---|---|---|
| | 358 sh. | 359 max. | 360 max.* | 365 sh. | 365 sh. |
| 408 max. | 414 max. | 423 max. | 416 max. | 413 max. | 411 max. |
| 502 max. | 542 max. | 540 max. | 534 max. | 533 max. | 533 max. |
| | 582 max. | | 560 sh. | 560 sh. | 560 sh. |
| | 595 sh. | | 630 sh. | 620 sh. | 620 sh. |

* This is a very shallow maximum

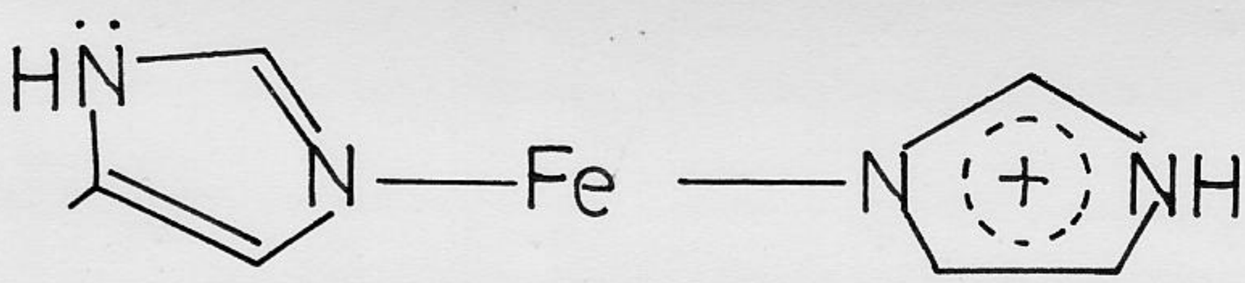
(1) Baghdoyan, (Thesis, 1965)

(2) Hanania et al., (1966)

E 7 histidine. The same suggestion can also be made in the case of horse Ac-Mb.




F 8



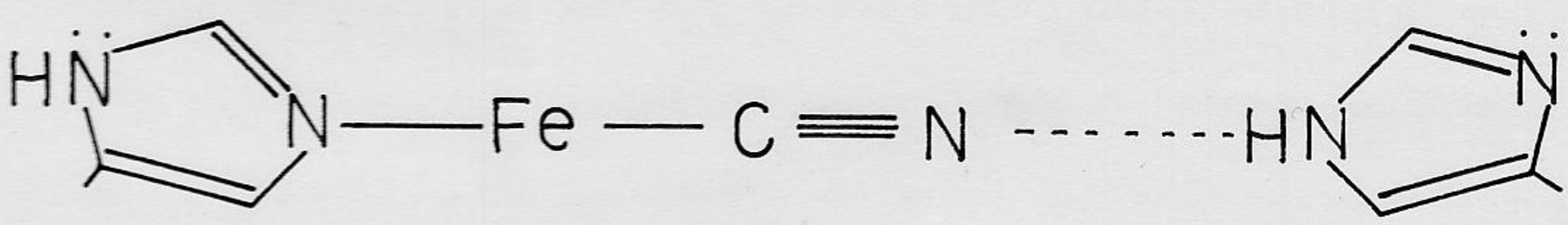

F8 E 7

The coordination of the iron atom to the E 7 histidine can be explained by a significant loss of the helical structure with an increased number of random coil segments in the modified myoglobin molecules. Evidence for this hypothesis is provided by the spectrophotometric titration of the tyrosine residues at 245 mμ (Fig. 14). It can be seen that in the modified myoglobins all three tyrosines are titrated, while in the case of the native myoglobins only 2 tyrosine residues are titrated; the third is not available below pH 12. Furthermore, the stability of the modified myoglobin molecule diminishes with increasing negative charge. In Fig. 12, it can be seen that Suc-Mb at pH 5.65 has a spectrum similar to that of Ac-Mb

and (n) Mb at pH 2.69. Similarly, at a pH 9.45, the absorption spectrum of Suc-Mb looks very much like the spectra of acetylated and native Mb at pH 12.02. Therefore, it can be seen that the degree of stability is related to the charge on the molecule. The limits of stability for Suc-Mb are narrower than for Ac-Mb, Suc-Mb is much more negatively charged than Ac-Mb. Similarly, the stability of Ac-Mb is less than the stability of (n) Mb, because all the positive charges on the molecule have been neutralized.

The ultraviolet spectrum of Ac-Mb, in the 280 mμ region, shows the loss of the 280 peak which is replaced by a continuous increase in absorption intensity. No ready explanation could be found for this. The cause of this spectral change cannot be attributed to acetyl tyrosine. O-acetyl groups will be hydrolyzed if kept at pH 10.8 for 15 min. (Atassi, 1966). Our samples of sperm whale and horse Ac-Mb were left in pH 11 for 2 hours and their spectra did not change. Moreover, the tyrosine titration can be seen very clearly in Fig. 14 and it is not time dependent.

Addition of cyanide to the modified myoglobins causes the formation of the iron-cyanide complex




F 8 E 7

since the binding of cyanide to the heme is much stronger than the binding of imidazole: for the heme-cyanide complex $K_{assoc.}$ = approx. $10^8$

(Nakhleh, Thesis 1968) while for the heme-imidazole complex $K_{assoc.}$ = approx. 170 (Baghdoyan, Thesis 1965). The more stable complex is formed and the imidazole group of the E 7 histidine is most probably hydrogen bonded to the cyanide group. The spectra of the cyanide complex of sperm whale (n) Mb and horse (n) Mb are almost insensible to changes in pH. But the spectra of the cyanide complex of the modified myoglobins exhibit definite changes with pH (Fig. 14: 360 mμ, 540 mμ). The absorption peaks at 540 mμ and 420 mμ in the spectra of sperm whale Ac-MbCN, sperm whale Suc-MbCN, and horse Ac-MbCN undergo a shift of 5 mμ to higher wavelengths accompanying a change in pH from 7.7 to 9.5 and the characteristic cyanide peak at 360 mμ changes its curvature in the same pH range. Moreover, the change in molar absorbance with pH at 540 mμ and 360 mμ can be seen in Fig. 14. This pH dependence may be due to electrostatic and steric perturbations in the environment of the heme. The variation of $\epsilon$ with pH cannot be explained by the dissociation of the cyanide complex, because the characteristic cyanide peak is always present in the spectra and even more accentuated at higher pH values. It can be seen that the change of $\epsilon$ with pH (Fig. 14) is more pronounced for sperm whale Suc-MbCN than for sperm whale Ac-MbCN, but less pronounced than for horse Ac-MbCN. This is probably due to the higher negative charge on horse (n) Mb molecule. A parallel can be established between this last point and the isoionic points of the three derivatives, measured by Sabri (Thesis, 1968): horse Ac-Mb, pI = 5.47; sperm whale Suc-Mb, pI = 5.96; sperm whale Ac-Mb, pI = 6.36; sperm whale (n) Mb, pH = 8.07; horse (n) Mb, pI = 7.44. The Soret band is also seen to be pH dependent.

In sperm whale Ac-MbCN, sperm whale Suc-MbCN and horse Ac-MbCN, the Soret peak undergoes a red shift of 5 mμ between pH 7.7 and 9.5. No major changes with pH variation are observed in the 320 - 270 mμ region.

The chromatography of sperm whale TC-Mb, on Sephadex G-75, revealed that the preparation had a molecular weight higher than 70,000, probably much higher, and could therefore be a covalent polymer of myoglobin. A high degree of polymerization can be explained, if we assume that the toluene-2,4-diisocyanate has modified more than one lysine residue per myoglobin molecule and that, during the second stage of the reaction, the covalent cross-linking of myoglobin molecules went beyond the dimer. The toluene-2,4-diisocyanate absorbs in the 280 mμ region due to the presence of the benzene ring. In the ultraviolet spectrum of TC-Mb, the sharp increase in absorption at around 280 mμ can be explained by the presence of an increased number of aromatic groups. Schick and Singer (1961) report the absorption spectrum of bovine serum albumin-m-xylylene diisocyanate in the 240 - 300 mμ region; this spectrum is very similar to that of TC-Mb in the same wavelength region. But further investigations are necessary to determine the exact structure of the modified TC-Mb.

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# APPENDIX

Abbreviations used:

## Table XII

G 0.05M glycine buffer

PB phosphate borax buffer

## Tables XIII - XV

Ac 0.05M acetate buffer, 0.1% KCN

P 0.05M phosphate buffer, 0.1% KCN

G 0.05M glycine buffer, 0.1% KCN

PB phosphate borax buffer, 0.1% KCN

Table VII

Characteristics of Absorption Spectra of Sperm Whale (n) Mb

Stock solution $2.01 \times 10^{-3}$ M

0.05M phosphate buffer pH 7.05

| Concentration, M | $\lambda$, mμ | A | $\epsilon \times 10^{-3}$, $M^{-1}\ cm^{-1}$ |
|---|---|---|---|
| $8.04 \times 10^{-5}$ | 630 max. | 0.280 | 3.48 |
| | 600 min. | 0.238 | 2.96 |
| | 580 max. | 0.278 | 3.46 |
| | 567 min. | 0.262 | 3.26 |
| | 535 sh. | 0.490 | 6.10 |
| | 502 max. | 0.710 | 8.84 |
| | 472 min. | 0.580 | 7.21 |
| $8.04 \times 10^{-6}$ | 408 max. | 1.187 | 148 |
| $3.22 \times 10^{-5}$ | 309 min. | 0.413 | 12.8 |
| | 286 sh. | 0.942 | 29.3 |
| | 280 max. | 1.042 | 32.5 |
| | 250 min. | 0.765 | 23.8 |

Table VIII

Characteristics of Absorption Spectra of Sperm Whale Ac-Mb

Stock solution $1.99 \times 10^{-3}$ M

0.05M phosphate buffer pH 7.05

| Concentration, M | λ, mμ | A | $\epsilon \times 10^{-3}$, $M^{-1}$ $cm^{-1}$ |
|---|---|---|---|
| $7.96 \times 10^{-5}$ | 620 sh. | 0.110 | 1.38 |
| | 560 sh. | 0.610 | 7.66 |
| | 533 max. | 0.750 | 9.43 |
| | 500 min. | 0.510 | 6.41 |
| | 485 sh. | 0.558 | 7.00 |
| $7.96 \times 10^{-6}$ | 412.5 max. | 0.790 | 99.4 |
| $3.18 \times 10^{-5}$ | 365 sh. | 0.830 | 26.1 |
| | 306 min. | 0.382 | 12.0 |
| | 288 sh. | 0.723 | 22.7 |
| | 278 sh. | 0.863 | 27.1 |
| | 262 sh. | 1.00 | 31.4 |
| | 256 sh. | 1.03 | 32.4 |

Table IX

Characteristics of Absorption Spectra of Sperm Whale Suc-Mb

Stock solution $1.69 \times 10^{-3}$

0.05M phosphate buffer pH 7.05

| Concentration, M | $\lambda$, mμ | A | $\epsilon \times 10^{-3}$, $M^{-1}$ $cm^{-1}$ |
|---|---|---|---|
| $6.76 \times 10^{-5}$ | 620 sh. | 0.110 | 1.63 |
| | 560 sh. | 0.430 | 6.36 |
| | 533 max. | 0.538 | 7.95 |
| | 500 min. | 0.403 | 5.96 |
| | 480 sh. | 0.450 | 6.65 |
| $6.76 \times 10^{-6}$ | 411 max. | 0.540 | 79.9 |
| $2.70 \times 10^{-5}$ | 365 sh. | 0.740 | 27.4 |
| | 305 min. | 0.320 | 11.8 |
| | 288 sh. | 0.610 | 22.6 |
| | 272 max. | 0.750 | 27.8 |
| | 260 sh. | 0.700 | 25.9 |
| | 254 min. | 0.665 | 24.6 |

Table X

Characteristics of Absorption Spectra of Horse (n) Mb

Stock solution $2.01 \times 10^{-3}$ M

0.05M phosphate buffer pH 7.05

| Concentration, M | $\lambda$, mμ | A | $\epsilon \times 10^{-3}$, $M^{-1}$ $cm^{-1}$ |
|---|---|---|---|
| $8.04 \times 10^{-5}$ | 630 max. | 0.280 | 3.48 |
| | 600 min. | 0.222 | 2.76 |
| | 590 max. | 0.225 | 2.80 |
| | 570 min. | 0.218 | 2.71 |
| | 540 sh. | 0.420 | 5.23 |
| | 502.5 max. | 0.718 | 8.94 |
| | 465 min. | 0.560 | 6.96 |
| $8.04 \times 10^{-6}$ | 408 max. | 1.24 | 154 |
| $3.22 \times 10^{-5}$ | 310 min. | 0.362 | 11.3 |
| | 288 sh. | 0.850 | 26.4 |
| | 280 max. | 0.960 | 29.8 |
| | 250 min. | 0.708 | 22.0 |

Table XI

Characteristics of Absorption Spectra of Horse Ac-Mb

Stock solution $1.75 \times 10^{-3}$ M

0.05M phosphate buffer pH 7.05

| Concentration, M | $\lambda$, mμ | A | $\epsilon \times 10^{-3}$, $M^{-1}$ $cm^{-1}$ |
|---|---|---|---|
| $7.00 \times 10^{-5}$ | 560 sh. | 0.572 | 8.18 |
| | 534 max. | 0.700 | 10.0 |
| | 500 min. | 0.438 | 6.25 |
| | 485 sh. | 0.490 | 7.00 |
| $7.00 \times 10^{-6}$ | 412.5 max. | 0.660 | 94.4 |
| $2.80 \times 10^{-5}$ | 360 sh. | 0.703 | 25.1 |
| | 305 min. | 0.320 | 11.4 |
| | 288 sh. | 0.637 | 22.8 |
| | 276 sh. | 0.777 | 27.8 |
| | 260 sh. | 0.920 | 32.8 |

Table XII

Characteristics of Absorption Spectra of Sperm Whale Ac-Mb

at Different pH Values

| $\lambda$, mμ | c x $10^5$ M | pH | Buffer | A | $\epsilon$ x $10^{-3}$, $M^{-1}$ $cm^{-1}$ |
|---|---|---|---|---|---|
| 610 sh. | 14.7 | 8.62 | G | 0.295 | 2.00 |
| | | 8.99 | G | 0.305 | 2.08 |
| | | 9.14 | G | 0.320 | 2.18 |
| | | 9.33 | G | 0.340 | 2.32 |
| | | 9.55 | G | 0.368 | 2.50 |
| | | 9.68 | G | 0.390 | 2.65 |
| | | 9.82 | G | 0.422 | 2.87 |
| | | 9.99 | G | 0.465 | 3.16 |
| | | 10.20 | G | 0.520 | 3.54 |
| | | 9.45 | PB | 0.382 | 2.60 |
| | | 9.95 | PB | 0.482 | 3.28 |
| | | 10.29 | PB | 0.622 | 4.24 |
| | | 10.90 | PB | 0.698 | 4.75 |
| | | 11.50 | PB | 0.740 | 5.04 |
| | | 12.02 | PB | 0.782 | 5.32 |
| 578 isb. | 14.7 | 8.62 - 10.20 | G | 0.820 ± 0.010 | 5.58 ± 0.07 |
| | | 9.45 - 12.02 | PB | 0.820 ± 0.010 | 5.58 ± 0.07 |
| 560 sh. | 14.7 | 8.62 | G | 1.142 | 7.79 |
| | | 8.99 | G | 1.155 | 7.86 |

Table XII Cont'd

| $\lambda$, mμ | c x $10^5$ M | pH | Buffer | A | $\epsilon$ x $10^{-3}$, $M^{-1}cm^{-1}$ |
|---|---|---|---|---|---|
| | | 9.14 | G | 1.132 | 7.72 |
| | | 9.33 | G | 1.132 | 7.72 |
| | | 9.55 | G | 1.110 | 7.49 |
| | | 9.68 | G | 1.078 | 7.34 |
| | | 9.82 | G | 1.050 | 7.15 |
| | | 9.99 | G | 1.012 | 6.90 |
| | | 10.20 | G | 0.970 | 6.60 |
| | | 9.45 | PB | 1.113 | 7.58 |
| | | 9.95 | PB | 1.022 | 6.97 |
| | | 10.29 | PB | 0.993 | 6.75 |
| | | 10.90 | PB | 0.905 | 6.15 |
| | | 11.50 | PB | 0.852 | 5.80 |
| | | 12.02 | PB | 0.815 | 5.55 |
| 532 max. | 14.7 | 8.62 | G | 1.365 | 9.30 |
| | | 8.99 | G | 1.385 | 9.44 |
| | | 9.14 | G | 1.370 | 9.32 |
| | | 9.33 | G | 1.358 | 9.24 |
| | | 9.55 | G | 1.338 | 9.10 |
| | | 9.68 | G | 1.290 | 8.77 |
| | | 9.82 | G | 1.257 | 8.54 |
| | | 9.99 | G | 1.205 | 8.20 |

Table XII Cont'd

| $\lambda$, mμ | c x $10^5$ M | pH | Buffer | A | $\epsilon$ x $10^{-3}$, $M^{-1}$ $cm^{-1}$ |
|---|---|---|---|---|---|
| | | 10.20 | G | 1.145 | 7.80 |
| | | 9.45 | PB | 1.330 | 9.05 |
| | | 9.95 | PB | 1.200 | 8.17 |
| | | 10.29 | PB | 1.143 | 7.80 |
| | | 10.90 | PB | 1.020 | 6.94 |
| | | 11.50 | PB | 0.942 | 6.40 |
| | | 12.02 | PB | 0.880 | 5.99 |
| 500 isb. | 14.7 | 8.62 - 10.20 | G | 1.010 ± 0.020 | 6.88 ± 0.14 |
| | | 9.45 - 12.02 | PB | 1.020 ± 0.010 | 6.94 ± 0.07 |
| 475 sh. | 14.7 | 8.62 | G | 1.138 | 7.74 |
| | | 8.99 | G | 1.157 | 7.87 |
| | | 9.14 | G | 1.160 | 7.90 |
| | | 9.33 | G | 1.165 | 7.93 |
| | | 9.55 | G | 1.170 | 7.97 |
| | | 9.68 | G | 1.175 | 8.00 |
| | | 9.82 | G | 1.180 | 8.04 |
| | | 9.99 | G | 1.190 | 8.10 |
| | | 10.20 | G | 1.202 | 8.20 |
| | | 9.45 | PB | 1.200 | 8.17 |
| | | 9.95 | PB | 1.220 | 8.30 |
| | | 10.29 | PB | 1.250 | 8.51 |

Table XII Cont'd

| $\lambda$, mμ | c x $10^5$ M | pH | Buffer | A | $\epsilon$ x$10^{-3}$, $M^{-1}$ $cm^{-1}$ |
|---|---|---|---|---|---|
| | | 10.90 | PB | 1.270 | 8.65 |
| | | 11.50 | PB | 1.280 | 8.71 |
| | | 12.02 | PB | 1.300 | 8.85 |
| 485 isb. | 14.7 | 8.62 - 10.20 | G | 1.52 ± 0.02 | 10.3 ± 0.14 |
| | | 9.45 - 12.02 | PB | 1.54 ± 0.01 | 10.5 ± 0.07 |
| 412 max. | 0.735 | 8.62 | G | 0.750 | 102 |
| | | 8.99 | G | 0.720 | 98.0 |
| | | 9.14 | G | 0.698 | 95.0 |
| | | 9.33 | G | 0.665 | 90.5 |
| | | 9.55 | G | 0.635 | 86.5 |
| | | 9.68 | G | 0.590 | 80.4 |
| | | 9.82 | G | 0.560 | 76.3 |
| | | 9.99 | G | 0.508 | 69.2 |
| | | 10.20 | G | 0.465 | 63.3 |
| 1.47 | | 9.45 | PB | 1.330 | 90.5 |
| | | 9.95 | PB | 1.060 | 72.2 |
| | | 10.29 | PB | 0.930 | 63.2 |
| | | 10.90 | PB | 0.770 | 52.4 |
| | | 11.50 | PB | 0.695 | 47.3 |
| | | 12.02 | PB | 0.660 | 44.9 |

Table XII Cont'd

| $\lambda$, mμ | c x $10^5$ M | pH | Buffer | A | $\epsilon$ x$10^{-3}$, $M^{-1}$ $cm^{-1}$ |
|---|---|---|---|---|---|
| 392 isb. | 0.735 | 8.62 - 10.20 | G | 0.410 ± 0.01 | 55.8 ± 0.15 |
| | 1.47 | 9.45 - 12.02 | PB | 0.810 ± 0.01 | 55.1 ± 0.70 |
| 360 sh. | 3.67 | 8.62 | G | 0.990 | 27.0 |
| | | 8.99 | G | 1.063 | 29.0 |
| | | 9.14 | G | 1.082 | 29.6 |
| | | 9.33 | G | 1.130 | 30.8 |
| | | 9.55 | G | 1.190 | 32.4 |
| | | 9.68 | G | 1.235 | 33.7 |
| | | 9.82 | G | 1.280 | 34.9 |
| | | 9.99 | G | 1.338 | 36.5 |
| | | 10.20 | G | 1.402 | 38.2 |
| | | 9.45 | PB | 1.260 | 34.4 |
| | | 10.95 | PB | 1.435 | 39.2 |
| | | 10.29 | PB | 1.482 | 40.4 |
| | | 10.90 | PB | 1.565 | 42.6 |
| | | 11.50 | PB | 1.590 | 43.3 |
| | | 12.02 | PB | 1.590 | 43.3 |
| 305 min. | 3.67 | 8.62 | G | 0.450 | 12.3 |
| | | 8.99 | G | 0.480 | 12.3 |
| | | 9.14 | G | 0.498 | 13.6 |
| | | 9.33 | G | 0.498 | 13.6 |

Table XII Cont'd

| $\lambda$, mμ | c x $10^5$ M | pH | Buffer | A | $\epsilon$ x$10^{-3}$, $M^{-1}$ $cm^{-1}$ |
|---|---|---|---|---|---|
| | | 9.55 | G | 0.520 | 14.2 |
| | | 9.68 | G | 0.535 | 14.6 |
| | | 9.82 | G | 0.555 | 15.1 |
| | | 9.99 | G | 0.575 | 15.7 |
| | | 10.20 | G | 0.600 | 16.4 |
| | | 9.45 | PB | 0.598 | 16.3 |
| | | 9.95 | PB | 0.660 | 18.0 |
| | | 10.29 | PB | 0.680 | 18.5 |
| | | 10.90 | PB | 0.717 | 19.5 |
| | | 11.50 | PB | 0.778 | 21.2 |
| | | 12.02 | PB | 0.840 | 22.9 |
| 288 sh. | 3.67 | 8.62 | G | 0.820 | 22.4 |
| | | 8.99 | G | 0.865 | 23.6 |
| | | 9.14 | G | 0.870 | 23.7 |
| | | 9.33 | G | 0.881 | 24.0 |
| | | 9.55 | G | 0.893 | 24.3 |
| | | 9.68 | G | 0.905 | 24.6 |
| | | 9.82 | G | 0.918 | 25.0 |
| | | 9.99 | G | 0.938 | 25.6 |
| | | 10.20 | G | 0.965 | 26.3 |
| | | 9.45 | PB | 0.995 | 27.1 |
| | | 9.95 | PB | 1.050 | 28.6 |

Table XII Cont'd

| $\lambda$, mμ | c x $10^5$ M | pH | Buffer | A | $\epsilon$ x$10^{-3}$, $M^{-1}$ $cm^{-1}$ |
|---|---|---|---|---|---|
| | | 10.29 | PB | 1.065 | 29.0 |
| | | 10.90 | PB | 1.100 | 30.0 |
| | | 11.50 | PB | 1.180 | 32.2 |
| | | 12.02 | PB | 1.245 | 33.9 |
| 278 sh | 3.67 | 8.62 | G | 0.962 | 26.2 |
| | | 8.99 | G | 1.010 | 27.6 |
| | | 9.14 | G | 1.020 | 27.8 |
| | | 9.33 | G | 1.040 | 28.4 |
| | | 9.55 | G | 1.045 | 28.5 |
| | | 9.68 | G | 1.060 | 28.9 |
| | | 9.82 | G | 1.072 | 29.3 |
| | | 9.99 | G | 1.090 | 29.7 |
| | | 10.20 | G | 1.120 | 30.5 |
| | | 9.45 | PB | 1.157 | 31.5 |
| | | 9.95 | PB | 1.203 | 32.8 |
| | | 10.29 | PB | 1.223 | 33.4 |
| | | 10.90 | PB | 1.260 | 34.3 |
| | | 11.50 | PB | 1.302 | 35.6 |
| | | 12.02 | PB | 1.330 | 36.2 |
| 245 | 3.67 | 8.62 | G | 1.390 | 37.9 |
| | | 8.99 | G | 1.440 | 39.2 |

Table XII Cont'd

| $\lambda$, mμ | c x $10^5$ M | pH | Buffer | A | $\epsilon$ x$10^{-3}$, $M^{-1}$ $cm^{-1}$ |
|---|---|---|---|---|---|
| | | 9.14 | G | 1.445 | 39.4 |
| | | 9.33 | G | 1.450 | 39.5 |
| | | 9.55 | G | 1.450 | 39.5 |
| | | 9.68 | G | 1.455 | 39.6 |
| | | 9.82 | G | 1.455 | 39.6 |
| | | 9.99 | G | 1.460 | 39.8 |
| | | 10.20 | G | 1.470 | 40.0 |
| | | 9.45 | PB | 1.550 | 42.2 |
| | | 9.95 | PB | 1.550 | 42.2 |
| | | 10.29 | PB | 1.550 | 42.2 |
| | | 10.90 | PB | 1.618 | 44.0 |
| | | 11.50 | PB | 1.800 | 49.0 |
| | | 12.02 | PB | 1.925 | 52.5 |

Table XIII

pH Profile of Sperm Whale (n) MbCN, Sperm Whale Ac-MbCN, Sperm Whale Suc-MbCN, Horse (n) MbCN and Horse Ac-MbCN at $\lambda$ = 540 mμ

| | cx10$^5$ M | pH | Buffer | A | $\epsilon$ x10$^{-4}$, M$^{-1}$ cm$^{-1}$ |
|---|---|---|---|---|---|
| Sperm Whale (n) MbCN | 8.04 | 5.60 | Ac | 0.840 | 1.04 |
| | | 5.97 | P | 0.840 | 1.04 |
| | | 7.05 | P | 0.840 | 1.04 |
| | | 7.71 | P | 0.840 | 1.04 |
| | | 9.52 | G | 0.840 | 1.04 |
| | | 9.99 | G | 0.840 | 1.04 |
| | | 10.60 | PB | 0.840 | 1.04 |
| | | 11.89 | PB | 0.825 | 1.02 |
| Sperm Whale Ac-MbCN | 7.96 | 5.60 | Ac | 0.842 | 1.06 |
| | | 5.97 | P | 0.835 | 1.05 |
| | | 7.05 | P | 0.853 | 1.07 |
| | | 7.71 | P | 0.862 | 1.08 |
| | | 9.52 | G | 0.871 | 1.09 |
| | | 9.99 | G | 0.880 | 1.11 |
| | | 10.60 | PB | 0.871 | 1.09 |
| | | 11.89 | PB | 0.850 | 1.07 |
| Sperm Whale Suc-MbCN | 6.76 | 5.60 | Ac | 0.588 | 0.870 |
| | | 5.99 | P | 0.600 | 0.889 |

Table XIII Cont'd

| | $cx10^5$ M | pH | Buffer | A | $\epsilon x10^{-4}$, $M^{-1}$ $cm^{-1}$ |
|---|---|---|---|---|---|
| | | 7.05 | P | 0.633 | 0.936 |
| | | 7.71 | P | 0.640 | 0.947 |
| | | 9.52 | G | 0.678 | 1.00 |
| | | 9.99 | G | 0.678 | 1.00 |
| | | 10.51 | PB | 0.670 | 0.992 |
| | | 11.89 | PB | 0.632 | 0.935 |
| Horse (n) MbCN | 8.04 | 5.60 | Ac | 0.815 | 1.01 |
| | | 5.99 | P | 0.815 | 1.01 |
| | | 7.05 | P | 0.815 | 1.01 |
| | | 7.71 | P | 0.815 | 1.01 |
| | | 9.52 | G | 0.815 | 1.01 |
| | | 9.99 | G | 0.815 | 1.01 |
| | | 10.51 | PB | 0.810 | 1.01 |
| | | 11.89 | PB | 0.810 | 1.01 |
| Horse Ac-MbCN | 7.00 | 5.60 | Ac | 0.765 | 1.09 |
| | | 5.99 | P | 0.750 | 1.07 |
| | | 7.05 | P | 0.770 | 1.10 |
| | | 7.71 | P | 0.800 | 1.14 |
| | | 9.52 | G | 0.896 | 1.28 |
| | | 9.99 | G | 0.890 | 1.27 |
| | | 10.51 | PB | 0.800 | 1.14 |
| | | 11.89 | PB | 0.878 | 1.25 |

Table XIV

pH Profile of Sperm Whale (n) MbCN, Sperm Whale Ac-MbCN, Sperm Whale Suc-MbCN, Horse (n) MnCN and Horse Ac-MbCN at $\lambda = 360$ mμ

| | cx$10^5$ M | pH | Buffer | A | $\epsilon$ x$10^{-4}$, $M^{-1}$ $cm^{-1}$ |
|---|---|---|---|---|---|
| Sperm Whale (n) MbCN | 3.22 | 5.60 | Ac | 0.910 | 2.83 |
| | | 5.97 | P | 0.910 | 2.83 |
| | | 7.05 | P | 0.910 | 2.83 |
| | | 7.71 | P | 0.910 | 2.83 |
| | | 9.52 | G | 0.910 | 2.83 |
| | | 9.99 | G | 0.910 | 2.83 |
| | | 10.60 | PB | 0.910 | 2.83 |
| | | 11.89 | PB | 0.910 | 2.83 |
| Sperm Whale Ac-MbCN | 3.18 | 5.64 | Ac | 0.880 | 2.76 |
| | | 5.97 | P | 0.852 | 2.68 |
| | | 7.08 | P | 0.852 | 2.68 |
| | | 7.72 | P | 0.880 | 2.76 |
| | | 9.52 | G | 0.955 | 3.00 |
| | | 10.00 | G | 0.960 | 3.02 |
| | | 10.53 | PB | 0.960 | 3.02 |
| | | 11.91 | PB | 0.945 | 2.97 |
| Sperm Whale Suc-MbCN | 2.70 | 5.60 | Ac | 0.778 | 2.88 |
| | | 5.99 | P | 0.742 | 2.74 |

Table XIV Cont'd

| | cx10$^5$M | pH | Buffer | A | $\epsilon$ x10$^{-4}$,M$^{-1}$ cm$^{-1}$ |
|---|---|---|---|---|---|
| | | 7.05 | P | 0.695 | 2.57 |
| | | 7.71 | P | 0.718 | 2.66 |
| | | 9.52 | G | 0.898 | 3.32 |
| | | 9.99 | G | 0.887 | 3.28 |
| | | 10.51 | PB | 0.840 | 3.10 |
| | | 11.89 | PB | 0.906 | 3.35 |
| Horse (n) MbCN | 3.22 | 5.60 | Ac | 0.890 | 2.77 |
| | | 5.99 | P | 0.890 | 2.77 |
| | | 7.05 | P | 0.900 | 2.80 |
| | | 7.71 | P | 0.900 | 2.80 |
| | | 9.52 | G | 0.900 | 2.80 |
| | | 9.99 | G | 0.900 | 2.80 |
| | | 10.51 | PB | 0.900 | 2.80 |
| | | 11.89 | PB | 0.900 | 2.80 |
| Horse Ac-MbCN | 2.80 | 5.60 | Ac | 0.800 | 2.86 |
| | | 5.99 | P | 0.780 | 2.78 |
| | | 7.05 | P | 0.800 | 2.86 |
| | | 7.71 | P | 0.830 | 2.96 |
| | | 9.52 | G | 1.01 | 3.61 |
| | | 9.99 | G | 1.00 | 3.57 |
| | | 10.51 | PB | 0.972 | 3.47 |
| | | 11.89 | PB | 1.00 | 3.57 |

Table XV

pH Profile of Sperm Whale (n) MbCN, Sperm Whale Ac-MbCN, Sperm Whale Suc-MbCN, Horse (n) MbCN and Horse Ac-MbCN at $\lambda = 245$ mμ

| | $cx10^5$ M | pH | Buffer | A | $\epsilon x10^{-4}$, $M^{-1}$ $Cm^{-1}$ |
|---|---|---|---|---|---|
| Sperm whale (n) MbCN | 3.22 | 5.60 | Ac | 1.04 | 3.24 |
| | | 5.97 | P | 1.06 | 3.30 |
| | | 7.05 | P | 1.06 | 3.30 |
| | | 7.71 | P | 1.10 | 3.42 |
| | | 9.52 | G | 1.17 | 3.64 |
| | | 9.99 | G | 1.17 | 3.64 |
| | | 10.60 | PB | 1.28 | 3.98 |
| | | 11.89 | PB | 1.58 | 4.91 |
| Sperm whale Ac-MbCN | 3.18 | 5.64 | Ac | 1.27 | 3.99 |
| | | 5.97 | P | 1.22 | 3.83 |
| | | 7.08 | P | 1.22 | 3.83 |
| | | 7.72 | P | 1.24 | 3.90 |
| | | 9.52 | G | 1.27 | 3.98 |
| | | 10.00 | G | 1.34 | 4.21 |
| | | 10.53 | PB | 1.45 | 4.55 |
| | | 11.91 | PB | 1.80 | 5.65 |
| Sperm whale Suc-MbCN | 2.70 | 5.60 | Ac | 0.740 | 2.74 |
| | | 5.99 | P | 0.760 | 2.81 |

Table XV Cont'd

| | cx10$^5$M | pH | Buffer | A | $\epsilon$ x10$^{-4}$, M$^{-1}$ cm$^{-1}$ |
|---|---|---|---|---|---|
| | | 7.05 | P | 0.780 | 2.88 |
| | | 7.71 | P | 0.820 | 3.03 |
| | | 9.52 | G | 0.880 | 3.26 |
| | | 9.99 | G | 0.950 | 3.52 |
| | | 10.51 | PB | 1.10 | 4.07 |
| | | 11.89 | PB | 1.57 | 5.80 |
| Horse (n) MbCN | 3.22 | 5.60 | Ac | 0.970 | 3.02 |
| | | 5.99 | P | 0.990 | 3.08 |
| | | 7.05 | P | 0.990 | 3.08 |
| | | 7.71 | P | 1.01 | 3.14 |
| | | 9.52 | G | 1.04 | 3.24 |
| | | 9.99 | G | 1.06 | 3.30 |
| | | 10.51 | PB | 1.12 | 3.48 |
| | | 11.89 | PB | 1.31 | 4.07 |
| Horse Ac-MbCN | 2.80 | 5.60 | Ac | 1.22 | 4.35 |
| | | 5.99 | P | 1.22 | 4.35 |
| | | 7.05 | P | 1.24 | 4.43 |
| | | 7.71 | P | 1.26 | 4.50 |
| | | 9.52 | G | 1.36 | 4.86 |
| | | 9.99 | G | 1.38 | 4.94 |
| | | 10.51 | PB | 1.56 | 5.57 |
| | | 11.89 | PB | - | - |

Table XVI

Characteristics of Absorption Spectra of Sperm Whale TC-Mb

Stock solution $2.14 \times 10^{-4}$ M

0.05M phosphate buffer pH 7.05

| Concentration, M | $\lambda$, mμ | A | $\epsilon \times 10^{-3}$, $M^{-1}cm^{-1}$ |
|---|---|---|---|
| $7.14 \times 10^{-5}$ | 620 sh. | 0.180 | 2.52 |
| | 555 sh. | 0.445 | 6.24 |
| | 525 max. | 0.575 | 8.06 |
| | 500 sh. | 0.530 | 7.44 |
| | 490 min. | 0.520 | 7.29 |
| | 480 sh. | 0.539 | 7.55 |
| $7.14 \times 10^{-6}$ | 409 max. | 0.679 | 95.2 |
| $7.14 \times 10^{-6}$ | 360 sh. | 0.207 | 29.0 |
| | 315 min. | 0.132 | 18.5 |
| | 286 sh. | 0.524 | 73.5 |
| | 252 sh. | 1.558 | 218 |
| | 242 sh. | 1.742 | 244 |