THE ION BINDING PROPERTIES OF CYTOCHROME C AND A STUDY OF A POSSIBLE INVOLVEMENT OF LYSINE RESIDUES

THE ION BINDING PROPERTIES OF CYTOCHROME C AND A STUDY OF A POSSIBLE INVOLVEMENT OF LYSINE RESIDUES

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KATJA PALCIC, Dipl. Ing.

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AUTHOR: Katja Palcic, Dipl. Ing. University of Ljubljana, Ljubljana, Yugoslavia.

SUPERVISOR: Dr. R. A. Morton

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ABSTRACT:

This thesis describes the ionic strength and ion binding effects on the oxidation reduction properties of cytochrome c and its lysine modified derivatives.

Cytochrome c has been modified in two different ways: a complete modification of all lysine residues and specific modification of one lysine residue. Some properties of the modified derivatives are described.

Ion binding properties of cytochrome c and its lysine modified derivatives were studied by measuring the apparent equilibrium constant of the reaction between the ferri- form of the protein and potassium ferrocyanide. It was found that unmodified cytochrome c binds one cation (K^+, Na^+) per molecule, and binding is much stronger

to the reduced form of the protein. Binding of cations is not changed upon modification of the lysine residues. For binding of the chloride, there are two binding sites on the cytochrome c molecule, and the binding is much stronger to the oxidized form of the protein. It was shown that upon the modification of the lysine residues in either way the binding of chloride was considerably changed. It was concluded that one of these two binding sites for chloride on cytochrome c involves lysine residue, probably the residue number 13.

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ABBREVIATIONS

A_λ:	absorbancy (optical density) of 1 cm path at wavelength λ
app:	apparent
cHo^{III}:	ferricytochrome c, from horse heart
cHo^{II}:	ferrocytochrome c, from horse heart
cm:	centimeters
ε_λ:	molar extinction coefficient at the wavelength λ
Gn-cHo:	guanidinated cytochrome c
I:	ionic strength
lys:	lysine
M:	molar
mg:	milligrams
ml:	milliliters
nm:	nanometers
ox:	oxidized
red:	reduced
T^+:	tris ion (see definition for "tris", p. xiii)
TFA:	trifluoroacetyl
TFA-cHo:	trifluoroacetylated cytochrome c
TNBS:	2,4,6-trinitrobenzene sulfonate
TNP:	2,4,6-trinitrophenyl

l-TNP-cHo: cytochrome c, trinitrophenylated on one lysine residue

ε-TNP-lys: lysine, trinitrophenylated on ε-NH_2 groups

TPCK-trypsin: L-(1-tosylamide-2-phe-yl) ethyl chloromethyl ketone treated trypsin

tris: (Hydroxymethyl) Aminomethane

trp: tryptophane

tyr: tyrosine

V: volts

I. INTRODUCTION

A. Structure and Function of Cytochrome c

Cytochrome c is a heme protein found in mitochondria of all aerobic organisms. The role of the mitochondria is to break down the energy rich molecules, obtained from foods, combine them with oxygen and store the energy produced in the form of ATP. Biological oxidations involve many enzymes, among them a chain of cytochromes, of which cytochrome c is a part (for review see Margoliash and Schejter, 1966; Dickerson, 1972).

Cytochrome c is an iron containing protein composed of one heme group and single polypeptide chain. The iron atom alternates between +2 and +3 oxidation state as the molecule interacts with cytochrome oxidase and cytochrome reductase, respectively. Cytochrome c is essentially present in all eukaryotic organisms including animals, plants and aerobic microorganisms. To date the primary structures from over 30 species have been determined, which enabled the study of the process of molecular evolution on this particular protein. Similarities in primary structure have been detected at different levels of complexity. One such similarity is the occurrence of identical residues at identical positions.

These occupy over one-third of the molecule (Nolan and Margoliash, 1968). A second type is the presence of so-called conservative substitutions. The amino acid at such a position varies but remains similar in size, shape and polarity, so that the chemical function at the position is conserved. A third kind among cytochrome c is in the tertiary folding of the molecule (Dickerson et al., 1971). All appear to be functionally identical in spite of considerable differences in amino acid sequence. Cytochrome c from all species will react with cytochrome oxidase from preparations of distantly related species (Dickerson et al., 1971).

B. Structure of Cytochrome c

The structure of crystalline horse heart ferricytochrome c has been determined by x-ray diffraction at 2.8 Å resolution by Dickerson et al. (1971). It is an example of one of the classical models of protein structure - oil drop. The polypeptide chain folds in aqueous solution so that its hydrophobic residues are buried in the interior, and the polar charged groups are on the surface of the molecule. A strong folding influence is the heme group which is also hydrophobic. The heme group is placed within a crevice and is covalently linked by cysteine 14 and cysteine 17* to the

*The numbering system used throughout this paper will be from the amino terminal of horse heart cytochrome c.

right* wall of the crevice. Four of the ligands of the iron atom are the four nitrogens of the porphirin structure, the fifth and sixth ligands are histidine 18 and methionine 80. One propionic side chain of the heme is in a polar environment at the surface of the molecule, the other one is deeply buried in the hydrophobic interior surrounding the heme and involved in a network of hydrogen bonds.

Acidic and basic groups are segregated on the molecular surface into two positively charged segments with a negatively charged segment between them. The reason for this is probably to enable the interaction of cytochrome c with cytochrome oxidase, cytochrome reductase and the mitochondrial membrane.

C. Conformational Change Upon Electron Transfer

Various approaches have been used to study the difference of cytochrome c structure in its two oxidation states. Ferro- and ferricytochrome c have different stabilities to pH, temperature, denaturing agents, enzymatic cleavage of peptide bonds, and have different circular dichroic spectra and hydrogen exchange properties (summarized by Margoliash and Schejter, 1966). All information suggests more compact structure of the reduced form of cytochrome c.

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*Right side is determined by position of residues numbers 1 to 47, and left by the position of residues 48 to 91 (Dickerson et al., 1971).

Recently Takano et al. (1971) succeeded in obtaining the structure of tuna ferrocytochrome from x-ray diffraction analysis at 2.45 Å resolution. This work has clarified the differences between structure of oxidized and reduced form of protein.

The features that are relatively constant in both oxidation states are the N-terminal helix (residues 1 to 11), the C-terminal helix (residues 89 to 91) and the heme group together with its covalently attached peptide (residues 12 to 18). A large change in conformation occurs at residue numbers from 77 to 83, the part of the molecule which contains the evoluationary invariant region from residues 80 to 70. This has been called a working heart of the molecule by Dickerson et al. (1971). There is also a minor change in the position of residues 19 to 25 which form a hairpin loop to the right of heme crevice, and which was suggested by Dickerson et al. (1971) to be responsible for binding to cytochrome oxidase.

D. The Role of Lysine Residues in Cytochrome c

There are 19 lysine and 2 arginine residues in horse heart cytochrome c, and only 12 acidic amino acid residues. The result is a basic protein with an isoelectric point near pH 10. Five of the lysine residues (numbers 27, 72, 73, 79 and 87) are invariable in all species for which sequences

are known (Dickerson et al., 1971). The others are usually replaced by another basic residue (arginine or histidine). The charged residues are not uniformly distributed along the molecule, but are clustered, and this distribution of charge is very important for activity of cytochrome c molecule with cytochrome oxidase. The site responsible for this interaction has been studied by chemically modifying the ε-NH_2 groups of the lysine residues. Modifications in which the positive charge of the group was retained (for example guanidination) do not inhibit the reactivity of cytochrome c with cytochrome oxidase (Hettinger and Harbury, 1964, 1965), whereas a significant loss of reactivity is observed when positive charge is removed. For example, trifluoroacetylation (Fanger and Harbury, 1965), acetylation (Wada and Okunuki, 1968) and trinitrophenylation (Wada and Okunuki, 1969; Sato et al., 1966) were all found to decrease the catalytic reactivity of cytochrome c, even though the conformation was not significantly altered (as judged from intrinsic viscosity measurements, activity of digestive enzymes, and the absorption spectra at neutral pH). This suggested that the positive charge of the lysine residues is essential in binding to cytochrome oxidase. When cytochrome c was acetylated, it was found that lysine 22 had the greatest reactivity, followed by lysine 13. The ability to react with cytochrome oxidase linearly decreased

with the number of acetyl groups incorporated. The complete loss of activity was found when 6 lysine residues were acetylated (Wada and Okunuki, 1968). The situation was different when lysine residues were modified by trinitrobenzene sulfonate. The most reactive residue was shown to be lysine 13, followed by lysine 22. In the preparations where only lysine 13 was modified the reactivity with cytochrome oxidase decreased by more than 50%.

If lysine 13 is essential for the cytochrome oxidase reaction a large decrease of activity should be observed with acetylated protein where only two residues were modified (residues 22 and 13), and this was not found. As shown by Dickerson et al. (1971), lysine 13 is placed on the outside of the molecule at the top end of the heme crevice. The acetyl group on lysine 13 is much less effective in inhibiting the reaction with cytochrome oxidase, than the trinitrophenyl group probably because the bulky hydrophobic TNP group partially blocks the entrance to the crevice, or prevents the re-arrangement of the polypeptide chain near the crevice upon the reduction.

E. Ion Binding by Cytochrome c

As discussed in section A, all cytochromes c appear to be functionally identical. The classical electron transport properties do not seem to provide a

basis to differentiate the various species, so an effort has been made to find other possible physiological functions of cytochrome c. One property shown to exhibit a large variation among cytochrome c from different species is the binding of various inorganic anions to ferricytochrome c (Barlow and Margoliash, 1966). An examination of ion binding properties has been made by Margoliash et al. (1970) by measurement of electrophoretic mobility of ferro- and ferricytochrome c in different ionic environments. The results indicate a correlation between ion binding properties of cytochrome c (Margoliash et al., 1970), and the transport properties of mitochondria (Klingenberg, 1970). On the basis of this correlation, it was suggested by Margoliash et al. (1970) that cytochrome c is a carrier for some of the substances which require a specific translocation mechanism to move in and out of the mitochondrial matrix.

F. The Purpose of This Work

In these experiments an attempt was made to examine quantitatively the binding of sodium, potassium and chloride ions to ferro- and ferricyotchrome c by measurement of the equilibrium constant. We have analyzed the derivatives containing modified lysine residues, to determine their possible role in ion binding.

Section 1 presents experiments done to determine some

of the properties of these lysine-modified derivatives. Such experiments are the perturbation spectra of chromophoric residues as a measure of alteration in conformation, and pH-titration to examine heme-associated, pH-induced transitions of the proteins.

Section 2 gives the results of the measurement of apparent equilibrium constants for the reaction of cytochrome c with ferrocyanide and the effect of ions. Differences in ion binding sites between oxidized and reduced forms of cytochrome c will alter the apparent equilibrium constant, and enable information about ion binding to be obtained. Similar experiments with the lysine modified derivatives of cytochrome c will suggest whether or not these groups play any role in the binding site of these ions.

The chemical modifications of lysine used in this work are (the structures at pH 7.0 are given):

(1) Trifluoroacetylation: $-NH_3^+ \rightarrow -\overset{H}{\underset{|}{N}}-\overset{O}{\overset{\|}{C}}-CF_3$

(2) Guanidination: $-NH_3^+ \rightarrow -\overset{H}{\underset{|}{N}}-\overset{NH}{\overset{\|}{C}}-NH_3^+$

(3) Trinitrophenylation: $-NH_3^+ \rightarrow -\overset{H}{\underset{|}{N}}-C_6H_3(NO_2)_2$ (with NO_2 at the 2- and 4-positions)

All are expected to be specific for the ε-amino group and the last one for lysine 13 under the reaction conditions used.

II. MATERIALS AND METHODS

A. Chemicals

(a) Horse heart cytochrome c, Sigma type III (Lot 70C 094D) was used in all experiments. For the modification reactions it was used without further purification. For the measurement of equilibrium constants, it was chromatographed on Amberlite^{R} CG-50, equilibrated with 0.02 M NH_4HCO_3, pH 8.3, using a linear concentration gradient to 0.4 M of the same buffer. A single peak was obtained from the column and its center was pooled. If necessary, the solution of cytochrome c was concentrated with ultrafiltration apparatus (Diaflo^{R}, Amicon Co.) through the membrane type UM-2. A small amount of $K_3Fe(CN)_6$ was added to the protein, to produce the completely oxidized form before it was dialyzed against tris-cacodylate buffer at the appropriate concentration.

(b) Crystalline cacodylic acid purchased from Sigma Chemical Co. was used without further purification. Purified grade obtained from Fisher Scientific Co. was crystallized from a mixture of 95% ethyl alcohol and ethyl ether, 3:1, chromatographed through a cation exchange column in the hydrogen form (Biorex 70), and recrystallized from the above mixture.

(c) Tris from Sigma Chemical Co., reagent grade, was used as such.

(d) All the other chemicals used were reagent grade and were used without further purification.

B. Modification Procedures

(a) Guanidinated cytochrome c was prepared according to the procedure of Hettinger and Harbury (1964). Cytochrome c at final concentration of 1% was added to a solution of O-methylisourea sulfate at a final concentration of 0.5 M and pH = 8.5. The pH was brought to pH = 11 with 4 M NaOH. The reaction mixture was kept at 4°C for 5 days (about 120 hours), then dialyzed against 0.01 M sodium phosphate buffer pH = 8.0. The modified protein was chromatographed on the ion exchange column (Amberlite^{R} CG-50), equilibrated with 0.01 M sodium phosphate buffer, pH 8.0, and eluted with a sodium chloride gradient to 0.5 M. A single peak was obtained from the column and its center was pooled, and concentrated by ultrafiltration. The concentrated protein was oxidized with an equimolar amount of $K_3Fe(CN)_6$ and dialyzed against tris-cacodylic acid buffer. Before the measurements of equilibrium constants, the guanidinated cytochrome c was filtered through a Sepadex G-100 column (equilibrated with 3×10^{-3} M tris-cacodylate, pH 7.0) in an attempt to remove traces of aggregated protein.

(b) Trifluoroacetylated cytochrome c was prepared according to the procedure of Fanger and Harbury (1965).

300 mg of cytochrome c, 7.5 ml H_2O and 3 ml of ethyltrifluoro-thioacetate was reacted at room temperature keeping the pH = 10.0 by titration with about 2.5 M KOH using automatic titration (Radiometer Type TTT II, and autoburette type ABU 11). After 70 min, an additional 1.2 ml of reagent was added, and the reaction was allowed to proceed another 60 min, then 0.2 N HCl was added to bring the pH to 8.0, and the solution filtered through a Sephadex G-25 column equilibrated with 0.01 M tris-HCl buffer, pH 7.5. The protein solution was concentrated by ultrafiltration, and purified by chromatography on Sephadex G-75, equilibrated with 0.01 tris-HCl buffer, pH 7.8. Two fractions were obtained from the column (as described by Fanger and Harbury), the second was pooled, then dialyzed against H_2O and lyophilized.

(c) Reaction of modified proteins with 2,4,6-trinitro-benzene sulfonate. Reaction with TNBS is specific for the primary amino group (Okuyama and Satake, 1960), and was used as a method for determining free amino groups in the protein. The method was developed by Habeeb (1966), and modified by Kakade and Liener (1969) in order to differentiate between free ε-amino groups of lysine residues, and α-NH_2 groups of the N-terminal amino acid. However, the N-terminal amino group in cytochrome c is naturally acetylated. The extent of modification of guanidinated cytochrome c and TFA-cytochrome c was examined as follows:

To 1 ml of solution containing about 1 mg of protein in 4% $NaHCO_3$, pH 8.5, was added 1 ml of a freshly prepared aqueous solution of 0.1% TNBS. The suspension was placed into a constant shaking bath at 40°C for 2 hrs. 3 ml of about 12 M HCl was added after the reaction was completed. The tubes were covered with inverted glass beakers and autoclaved at 120° (15-17 psi) for 1 hr. After the hydrolysate had cooled to room temperature, 5 ml of distilled water was added to each tube. Solutions were extracted 2X with about 10 ml of ethyl ether in order to remove α-TNP groups. Residual ether was eliminated by placing tubes into hot water for 5 min. The absorbances of the solutions were measured against a blank at 345 nm. The blank was carried through the same procedure, except that HCl was added before the TNBS. The amount of ε-TNP lysine was calculated by using $\varepsilon_{345} = 1.46 \times 10^4\ M^{-1}\ cm^{-1}$ (Kakade and Liener, 1969). This figure takes into account approximately a 6% loss of TNP-lysine during hydrolysis.

(d) Trinitrophenylation of cytochrome c. Reaction of lysine residues in cytochrome c with TNBS has been described by several authors (Ando et al., 1965; Sato et al., 1966). Wada and Okunuki (1969) described the specific modification with TNBS of reactive lysine residues in bovine heart cytochrome c. We modified their procedure to prepare mono ε-TNP cytochrome c from horse heart. Cytochrome c was reacted with TNBS in an equimolar ratio to the ε-amino

groups (i.e., the molar concentration was nineteen times the molar concentration of cytochrome c). The reaction was carried out in 0.1 M sodium phosphate buffer, pH 7.2, at 4°C for 60 min. The reaction mixture was then passed into a Sephadex G-25 column, equilibrated with 0.02 M sodium phosphate buffer, pH 7.2, in order to remove the excess TNBS reagent. The protein solution eluted from the G-25 column was adsorbed to an Amberlite CG-50 column equilibrated at 4°C with 0.02 M sodium phosphate buffer, pH 7.2, and subsequently eluted with a sodium chloride gradient to 0.5 M. Fractions obtained from the column were examined for the extent of modification spectrophotometrically by using the extinction coefficient of ε-TNP-lys at 345 nm, $\varepsilon_{345} = 14.5 \times 10^3\ M^{-1}\ cm^{-1}$ (Wada and Okunuki, 1969). The average number of ε-TNP groups on the protein was calculated using the expression:

$$\bar{N} = \frac{A_{345}^{ox} - 0.85\ A_{550}^{red}}{A_{550}^{red}} \times \frac{27.7}{14.5}$$

Fractions with $\bar{N}$ approximately equal to 1 were re-chromatographed twice on the same type of CG-50 column with a sodium chloride gradient to 0.35 M in order to obtain single peak with $\bar{N} = 1$. Absorbancy profiles of the chromatography are given in the RESULTS section.

C. Perturbation Spectra of Cytochrome c and Lysine-Modified Proteins in 20% Ethylene Glycol

The absorption spectrum of chromophoric residues which have access to solvent may be sensitive to the changes in physical properties of the solvent. This can be used as a method of probing the surface of a protein molecule. In principle, only the groups located on or near the surface of the molecule should experience the perturbing effect of solvent changes. Solvent perturbation spectra have been studied with several proteins as a method of locating tryptophane and tyrosine residues (Herskovits and Laskowsky, 1962, Herskovits, 1969).

The difference spectral measurements were carried out with a Cary Model 14 spectrophotometer, by using a pair of cuvets with split path. Each cell has two compartments, one for protein solution and one for perturbant (ethylene glycol). The base line was determined when both cuvets contained 1 ml of protein solution, and 1 ml of 40% ethylene glycol in each compartment of the cell. The difference spectra were then obtained by mixing the solutions in one of the cuvets. The proteins were dissolved in 0.1 M potassium phosphate buffer, pH 7.0, at a concentration of 2 mg per ml. The 40% ethylene glycol solution in the same buffer was adjusted to pH 7.0. The difference spectra were measured from 240 to 350 nm, the region of tyrosine and tryptophane absorption.

D. Spectrophotometric pH Titrations

Spectrophotometric pH titrations were made using a Unicam Model SP1800 equipped with thermostalled cell compartment. The temperature was maintained at 25 ± 0.5°C. The pH was measured with Radiometer Model 28 pH meter, and electrode type G222. Proteins were dissolved in 0.01 - 0.005 M cacodylate-KOH buffer, pH 6.7 and were kept oxidized with a small amount of $K_3Fe(CN)_6$. Small amounts of 2 M KOH were added as a titrant with a microburet.

E. Measurements of the Equilibrium Constant

The apparent equilibrium constant for the reaction between ferricytochrome c and ferrocyanide was measured spectrophotometrically, using Unicam Model SP1800 at temperature 25°C.

Successive portions of ferricytochrome c solutions were added to a cuvet containing buffer with varying tris-cacodylate concentrations with or without additional anions or cations. The absorbancy at 550 nm of the completely oxidized protein was measured. Then the ferrocyanide was added to the solutions and the absorbancy at the same wavelength was measured after each addition. The total concentration of protein was obtained in separate measurements from the absorbancy at 550 nm, measured after addition of solid potassium ferricyanide, and sodium dithionite to the

samples. Difference in extinction coefficients between completely oxidized form and completely reduced form was taken as $\Delta\varepsilon_{550} = 1.85 \times 10^{-4}\ M^{-1}\ cm^{-1}$. Buffers of variable ionic strength were obtained by increasing the concentration of tris-cacodylate and keeping pH=7.0 constant. Concentrations of tris-cacodylate were used in the range from 3×10^{-3} to 7×10^{-3} M.

Buffers of variable concentration of binding cations (K^+, Na^+) were obtained by keeping the concentration of anion (cacodylate) constant, and adding KOH (NaOH) in the concentration range from 1 to 8×10^{-4} M to the solutions. The pH was brought to 7.0 with tris.

Buffers of variable concentration of binding anion (Cl^-) were prepared by keeping the concentration of cation (tris) constant and adding HCl in the concentration range 2 to 8×10^{-4} M. The pH was brought to 7.0 with cacodylic acid.

Temperature in all experiments was kept constant at 25°C ± 0.5.

III. EXPERIMENTAL RESULTS AND DISCUSSION

SECTION I. Properties of Modified Cytochrome c

A. The Extent of Modification of Guanidinated Cytochrome c and Trifluoroacetylated Cytochrome c

The extent of modification of cytochrome c with O-methylisourea and S-ethyl trifluoroacetate was tested by reacting the products with TNBS as described under MATERIALS AND METHODS. Table 1 shows the results obtained for unmodified cytochrome c, guanidinated cytochrome c and TFA cytochrome c under the same conditions.

TABLE 1

Determination of Free Amino Groups in Cytochrome c and Modified Derivatives Using Trinitrobenzene sulfonate

Cytochrome c	$\bar{N}$	$\bar{N}$/19x100
unmodified	15.3 ± 0.5	80.5
guanidinated cytochrome c	0	0
TFA cytochrome c	1.1 ± 0.1	5.8

$\bar{N}$ is the average number of TNP-lysine residues obtained per mole of protein. The amino acid sequence of horse heart cytochrome c contains 19 lysine residues.

We were never able to obtain the complete trinitrophenylation of unmodified cytochrome c. The error is possibly due to hydrolysis during the procedure or incomplete reaction.

No reaction of TNBS with guanidinated cytochrome c suggests that all 19 lysines are modified, as observed by Hettinger and Harbury (1964).

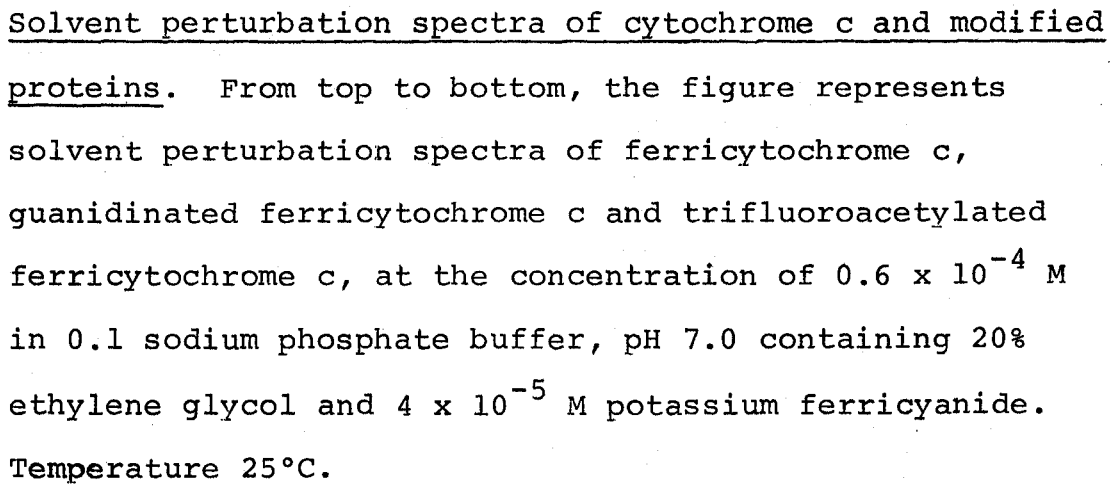
The result of TNBS reaction with TFA-cytochrome c indicates that trifluoroacetylation does not completely modify all 19 lysines. This is in disagreement with Fanger and Harbury (1965). However the trifluoroacetyl group is more readily hydrolyzed than homoarginine, and so it is possible that some were lost during the preparation or analysis of the TFA-cytochrome c.

B. Perturbation Spectra of Cytochrome c, Guanidinated Cytochrome c and Trifluoroacetylated Cytochrome c in 20% Ethylene Glycol

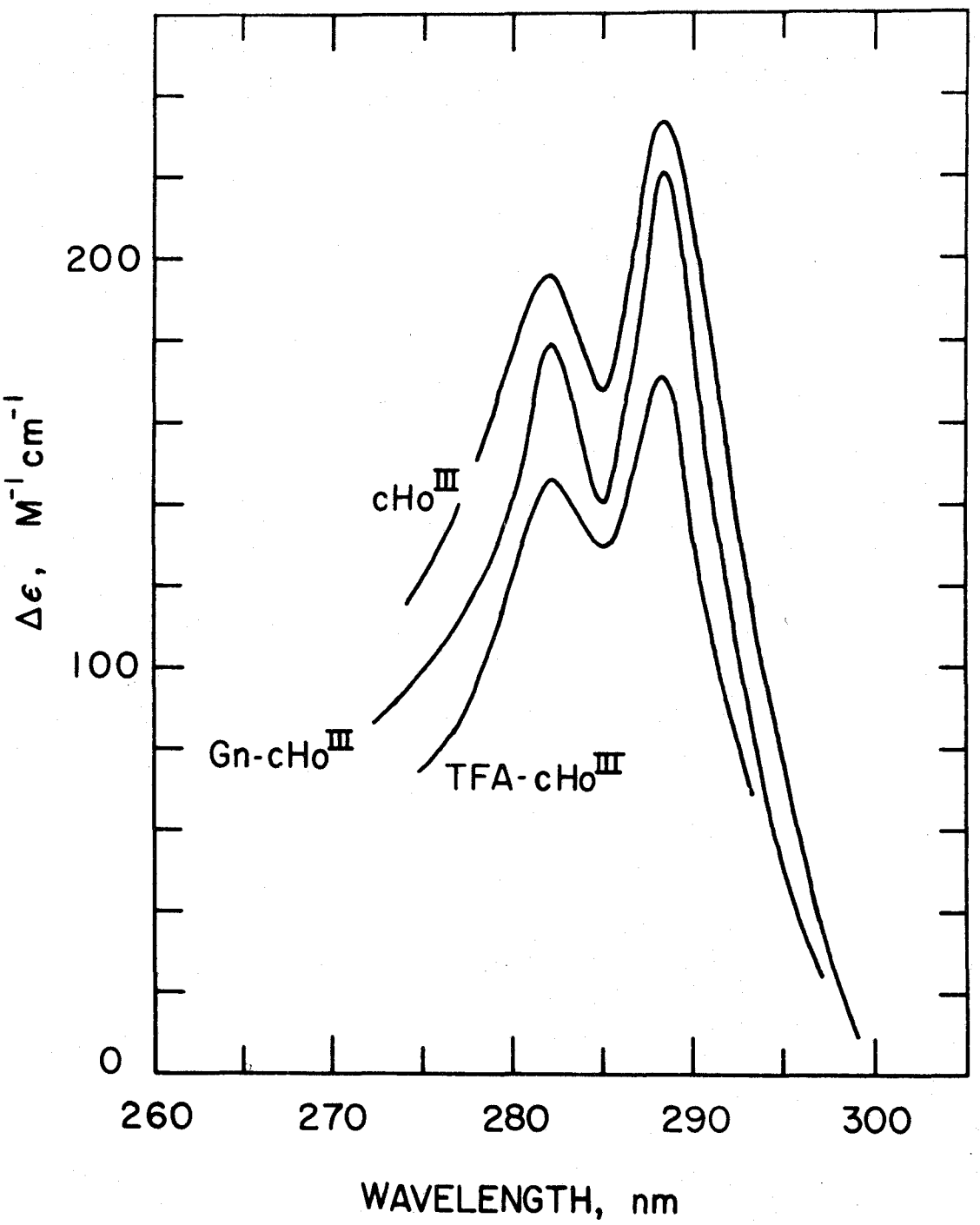
The solvent perturbation method has been employed as probe for locating chromophoric residues in proteins. For cytochrome c and its derivatives it was possible to estimate the fraction of exposure of tryptophane and tyrosine residues to the solvent. Figure 1 shows the perturbation spectra of the proteins in 20% ethylene glycol at pH 7.0.

In order to obtain curves of Fig. 1, the baseline was extrapolated from longer wavelengths in order to correct for the approximately constant contribution of the heme

FIGURE 1



Solvent perturbation spectra of cytochrome c and modified proteins. From top to bottom, the figure represents solvent perturbation spectra of ferricytochrome c, guanidinated ferricytochrome c and trifluoroacetylated ferricytochrome c, at the concentration of 0.6×10^{-4} M in 0.1 sodium phosphate buffer, pH 7.0 containing 20% ethylene glycol and 4×10^{-5} M potassium ferricyanide. Temperature 25°C.


200
100
0
Δε, M⁻¹cm⁻¹
cHo^III
Gn-cHo^III
TFA-cHo^III
260
270
280
290
300
WAVELENGTH, nm

group in the region from 250 to 300 nm (see Herskovits, 1969). To estimate the number of residues accessible to the perturbing solvent, a model compound which is fully exposed is required. The comparison has been made with N-acetyl esters of tyrosine and tryptophane (both amino and carboxyl groups blocked) by Herskovits and Sorensen (1968A), and additivity of tyrosyl and tryptophyl contributions to the spectra have been shown. By employing the equations (Herskovits and Sorensen, 1968B):

$$\Delta\varepsilon_{291} = a\Delta\varepsilon_{292}^{trp} + b\Delta\varepsilon_{292}^{tyr}$$

$$\Delta\varepsilon_{288} = a\Delta\varepsilon_{285.5}^{trp} + b\Delta\varepsilon_{285.5}^{tyr}$$

it is possible to obtain a and b which represent the apparent number of exposed tryptophane and tyrosine residues, respectively, in the protein. $\Delta\varepsilon_{\lambda}$ refers to the molar extinction coefficient differences of the protein, and free tyrosine and tryptophane model compounds. The model compound data necessary for the calculation have been taken from the same reference ($\Delta\varepsilon_{292}^{trp} = 305.1$; $\Delta\varepsilon_{292}^{tyr} = 16.1$; $\Delta\varepsilon_{285.5}^{trp} = 172.2$; $\Delta\varepsilon_{285.5}^{tyr} = 92.1$). Table 2 shows the values a and b calculated from the spectra. Herskovits (1969) estimated the exposure of the single tryptophane residue in cytochrome c as 55 to 70%. However, x-ray diffraction data by Dickerson et al. (1970) show that the tryptophane residue is buried in the hydrophobic

TABLE 2

Number of Exposed Tryptophane and Tyrosine Residues to 20% Ethylene Glycol in Cytochrome c and Modified Derivatives

Protein	a exposed tryptophane	b exposed tyrosine
cHo^{III}	0.47	1.6
Gn-cHo^{III}	0.45	1.5
TFA-cHo^{III}	0.29	1.3

environment, suggesting that it cannot be exposed to the solvent perturbation effect to such an extent. The same data show that out of 4 tyrosine residues in cytochrome c, two are completely buried (res. 48 and 67). The other two extend their hydroxyl groups out into the surrounding medium, suggesting that two tyrosine residues are exposed to the solvent perturbation effect.

From Fig. 1 and Table 2 we can see that guanidination hardly affects the surface of the cytochrome c molecule at all, whereas trifluoroacetylation affects the structure slightly more. This is perhaps not surprising if we consider that overall charge of the molecule is significantly changed upon this modification.

C. Preparation and Properties of 1-TNP-Cytochrome c.

When cytochrome c was treated with TNBS for a short time at 4°C as described in MATERIALS AND METHODS, the excess of reagent removed on G-25 column, the resulting modified protein had an average of 1.1 TNP residues per molecule. However, it was composed of a mixture of derivatives with 0, 1, 2 and 3 TNP groups per molecule.

The first purification step on the ion exchange column, CG-50, separated this mixture into several fractions (Fig. 2a).

Fraction II (tubes 80-100) was pooled together, dialyzed against 0.02 M sodium phosphate buffer, pH 7.2. The dialyzed solution was subjected to the chromatography on the same column, elution was obtained with linear concentration gradient of NaCl to 0.35 M. The mixture was separated into 3 fractions and the fraction with average number of TNP per molecule about 1 was re-chromatographed for the third time (Fig. 2b). In the third purification step on CG-50 column, a single peak was obtained and its center pooled (tubes 54-62). The resulting modified protein contained 1.00 TNP group per molecule in average.

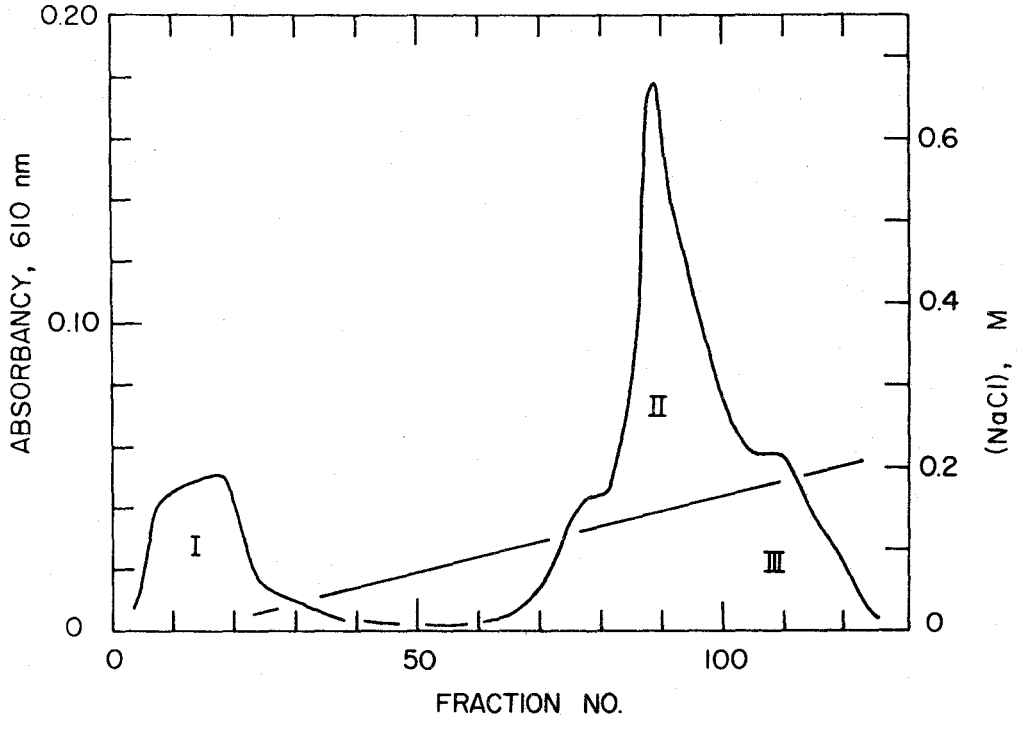
In conjunction with R. A. Morton (unpublished) the analysis of the 1-TNP-cytochrome was made. About 20 mg of the purified preparation of the modified protein was digested with TPCK trypsin at pH 8.4. The hydrolysate

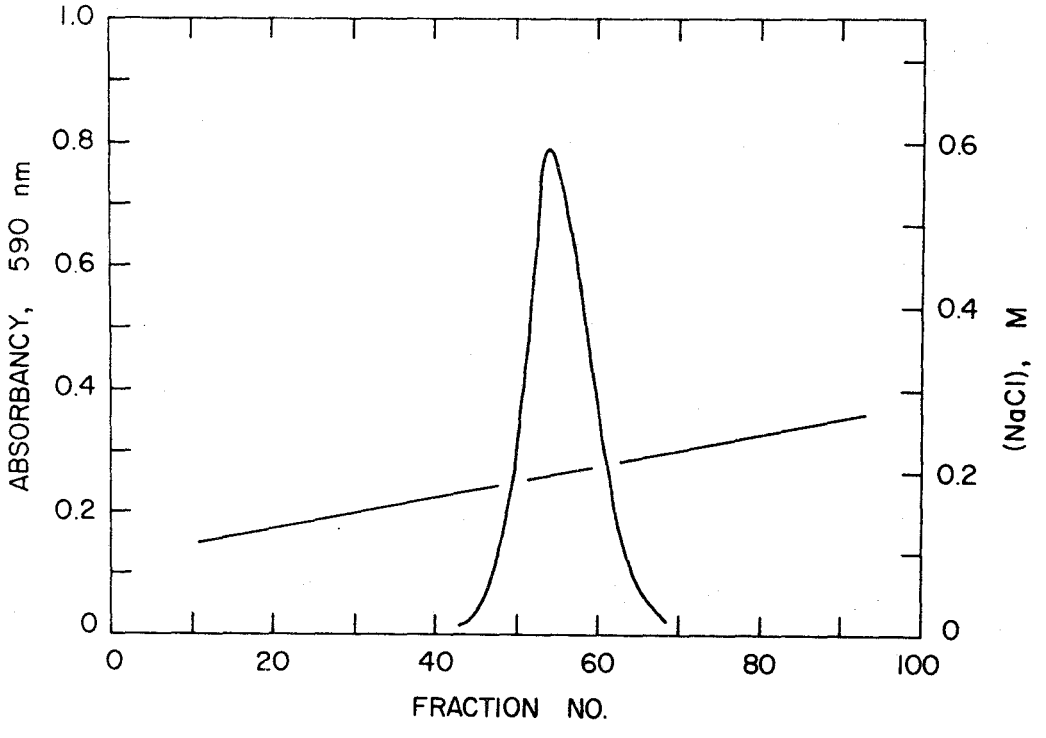
FIGURE 2

Elution Profile of 1-TNP-cHo on CG-50 Column Chromatography.

A. Column chromatography of TNP-cHo^{III} on a CG-50 column equilibrated at 4°C with 0.02 M sodium phosphate buffer pH 7.2, and eluted with NaCl gradient to 0.5 M. The unabsorbed fraction contained more than 2 TNP groups per molecule. Fraction II had $\bar{N}$ = 1.5 - 1.0 groups per molecule and Fraction III had $\bar{N} < 0.1$.

B. Fraction II from Fig. 2A (tubes 80 to 100 from A) was rechromatographed a third time on CG-50, equilibrated with the same buffer. As eluent, an NaCl gradient was used to a final concentration 0.35 M. The average number of TNP groups per molecule was measured as 1.4, 1.05, 0.9 in fractions 50, 54 and 63 and fractions 52 to 63 were pooled and used in this work.


0.20
0.10
0
ABSORBANCY, 610 nm
0.6
0.4
0.2
0
(NaCl), M
I
II
III
0
50
100
FRACTION NO.

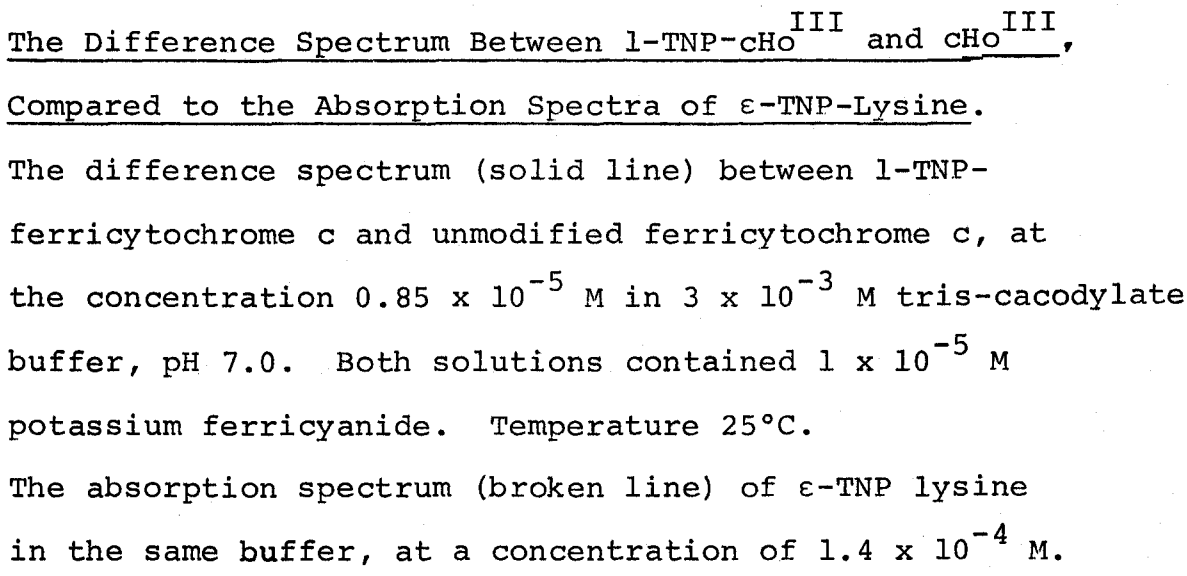

1.0
0.8
0.6
0.4
0.2
0
ABSORBANCY, 590 nm
0.6
0.4
0.2
0
(NaCl), M
0
20
40
60
80
100
FRACTION NO.

was examined by paper chromatography for the heme containing peptide, and compared with tryptic peptide from unmodified cytochrome c. The chromatography showed that the heme containing peptide was different in hydrolysate obtained from 1-TNP-cHo, as would be expected if lysine 13 was blocked and so prevented the cleavage at this point. The result is consistent with the predominant modification of 1-TNP-cytochrome c being at position number 13, the same residue modified in bovine cytochrome c (Wada and Okunuki, 1969).

Spectral properties of 1-TNP-cytochrome c: The difference in absorption spectrum between native ferricytochrome c and 1-TNP-ferricytochrome c was observed in the region of wavelengths shorter than 500 nm and is given in Figure 3. For comparison the absorption spectra of ε-TNP lysine is given. ε-TNP lysine was prepared following the procedure of Okuyama and Satake (1960).

The difference between ε_λ for ε-TNP lysine and the difference spectrum between the two proteins may be partly due to the fact that the TNP group in 1-TNP-cytochrome c is in a different environment than TNP-lysine in aqueous solution. The most significant difference between the two spectra is at $\lambda \approx 410$ nm, and may be due to the difference in heme absorption between the two proteins. However, Figure 3 indicates at most only small perturbation of heme group by TNP groups attached to lysine 13 (the order of 10%).

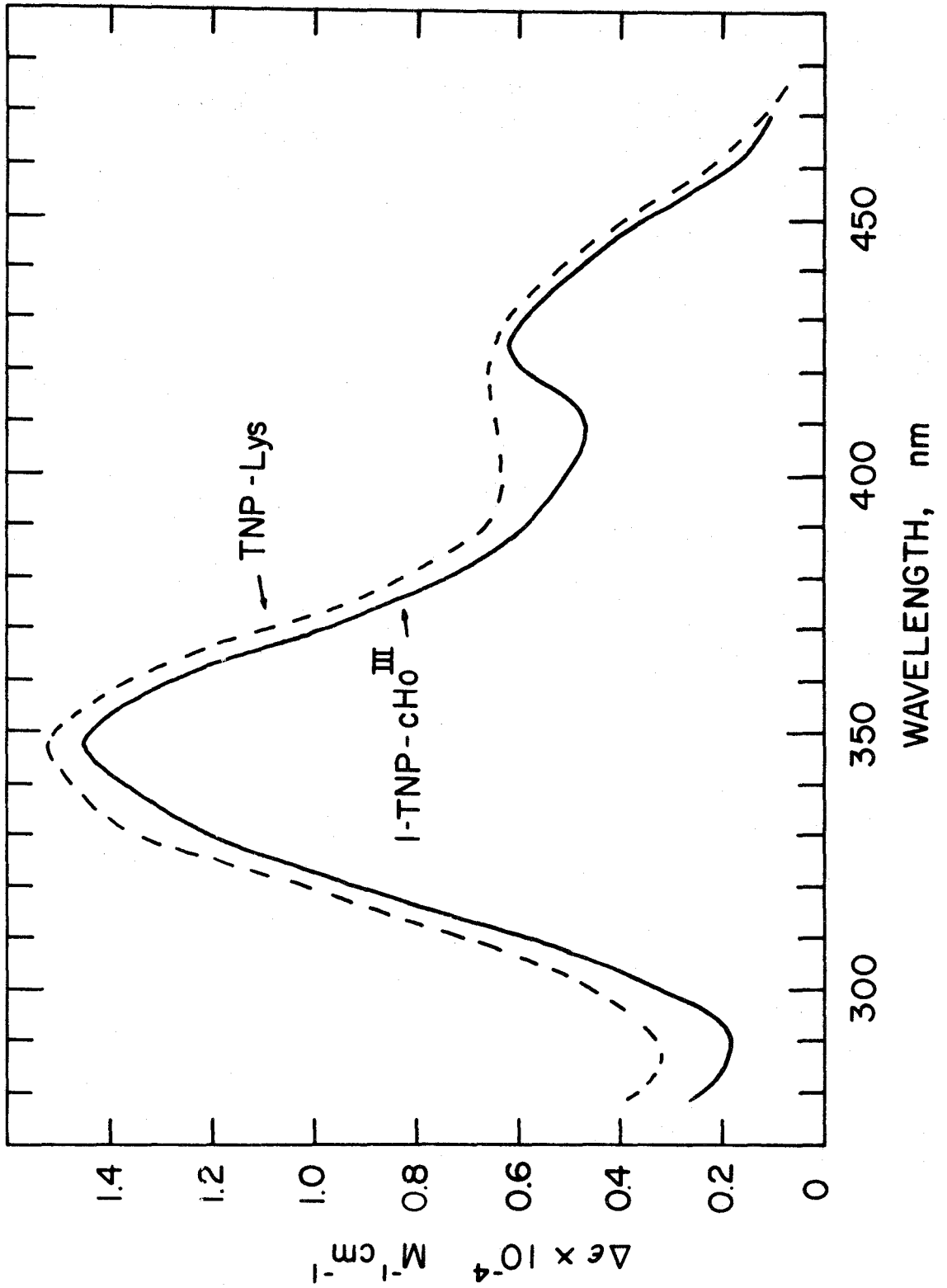
FIGURE 3



The Difference Spectrum Between 1-TNP-cHo^{III} and cHo^{III}, Compared to the Absorption Spectra of ε-TNP-Lysine.

The difference spectrum (solid line) between 1-TNP-ferricytochrome c and unmodified ferricytochrome c, at the concentration 0.85×10^{-5} M in 3×10^{-3} M tris-cacodylate buffer, pH 7.0. Both solutions contained 1×10^{-5} M potassium ferricyanide. Temperature 25°C.

The absorption spectrum (broken line) of ε-TNP lysine in the same buffer, at a concentration of 1.4×10^{-4} M.

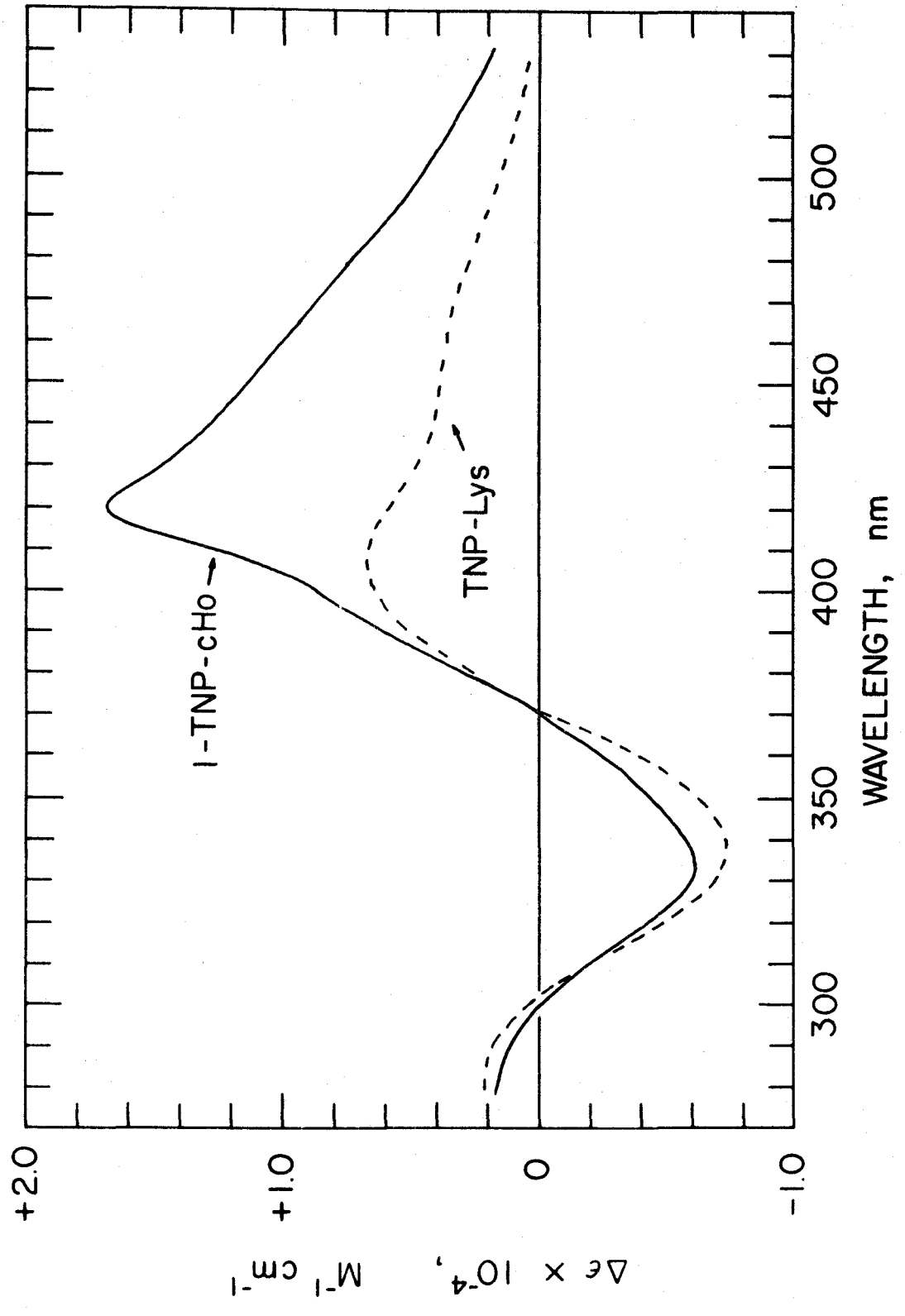

TNP-Lys
I-TNP-cHo III
WAVELENGTH, nm
300
350
400
450
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
Δε x 10⁻⁴ M⁻¹cm⁻¹

The difference spectrum of 1-TNP-ferrocytochrome c was not obtained, because of the reactivity of the TNP group. Sokolovsky et al. (1967) have shown that nitrotyrosine is reduced to amino tyrosine by sodium dithionite. The same reduction took place with 1-TNP-cHo^{III}. This reaction was observed to be more favourable than the reduction of the heme group. Figure 4 gives the difference spectra of reduced 1-TNP-cHo^{III} (reductant sodium dithionite) and cHo^{II} compared to the difference spectra of reduced and oxidized ε-TNP lysine.

The added amount of sodium dithionite of 1-TNP-cHo^{III} was not sufficient to reduce the heme group of the protein. There was no α band at 550 nm observed, after the addition. The difference of the absorption spectra at $\lambda \approx 420$ nm does not appear to be due to the reduction of cHo^{III} in one side of the spectrophotometer.

FIGURE 4

The Spectrum of the Reduced TNP Group in 1-TNP-cHo Compared with Reduced TNP-Lysine. The difference spectrum (solid line) was obtained by placing two cuvets in series on each side of the spectrophotometer. One cuvet of each pair in series contained 1-TNP-cHo^{III}, the other cHo^{III}, both of a concentration of 0.90×10^{-5} M (A_{530} identical) in 3×10^{-3} M tris-cacodylate pH 7.0. Temperature 25°C. The base line was then drawn. The difference spectrum was measured after addition of small amount of sodium dithionite to the sample side of 1-TNP-cHo^{III}, and the reference side of the cHo^{III} solution. The difference spectrum (broken line) between ε-TNP lysine reduced with sodium dithionite reduced and the oxidized form in the same buffer as above.


+2.0
+1.0
0
-1.0
300
350
400
450
500
WAVELENGTH, nm
Δε x 10⁻⁴, M⁻¹ cm⁻¹
1-TNP-cHo
TNP-Lys

D. The Alkaline pH Titration of 1-TNP-Cytochrome c.

Alkaline pH titration of unmodified cytochrome c shows the conversion of type III (the native form of the molecule at pH 7) to type IV (predominant form above pH 9) as defined by Theorell and Åkesson (1941). This conversion has been attributed to replacement of methionine 80 by lysine 79 as the iron ligand in type IV (Redfield and Gupta, 1971). Alkaline pH titration of native ferricytochrome c, guanidinated and trifluoroacetylated derivatives have been studied by R. A. Morton (to be published). Both lysine modified derivatives show different pH-induced transitions from native cytochrome c. Neither has the characteristic type III to type IV conversion. The spectral changes observed for Gn-cHo^{III} and TFA-cHo^{III} have been explained by the conversion to a complex in which a hydroxide ion replaced methionine 80 as the 6th ligand at alkaline pH, resulting in a thermal equilibrium between high-spin and low-spin spectral types (R. A. Morton, to be published). The e.s.r. studies of these two derivatives show that Gn-cHo^{III} retains the same electronic configuration of the iron atom, while this is not the case with TFA-cHo^{III}, indicating that positive charge of the molecule is important in maintaining the configuration of the protein (R. A. Morton, to be published).

In this study a derivative of cHo^{III} has been prepared in which only a single lysine residue has been

modified - probably lysine 13. It was of interest to determine whether or not modification of this lysine prevents, or modifies the conversion of type III to type IV.

The characteristic change in absorption spectrum upon the transition from type III to type IV in unmodified cytochrome c is the loss of the 695 nm absorption band. The derivatives with essentially all lysine groups modified show instead a strong absorption band at about 600-610 nm, characteristic of a contribution from a high-spin heme-iron complex. Figure 5 gives the difference spectra for alkaline pH titration of 1-TNP-cHo^{III}. The characteristic loss of the 695 nm absorption band is observed from which we can conclude that methionine 80 is displaced as the 6th ligand at alkaline pH.

Since lysine 13 is modified and presumably unable to coordinate, this result means that this residue is not the one which replaces methionine in 1-TNP-cHo^{III} and therefore probably not the one involved in the reaction in unmodified cytochrome c. The difference spectra of Figure 5 show that the protein complex found at alkaline pH is still low spin (absence of 600 to 620 nm absorption band which would give a positive peak in the difference spectra). The results are consistent with lysine 79 replacing methionine 80 as the 6th ligand.

The alkaline pH induced transition was further examined

FIGURE 5

Alkaline pH Titration of 1-TNP-cHo^{III} in the Region from 450 to 730 nm. The pH-induced difference spectra of 1-TNP-cHo^{III} in 3 x 10^{-3} M tris-cacodylate buffer, at the concentration of 1.22 x 10^{-4} M. Solutions contained 10^{-5} M potassium ferricyanide. Temperature 25°C. Reference pH was 7.23 and the pH measured were: a, 10.53; b, 9.61; c, 8.60; d, 7.96.

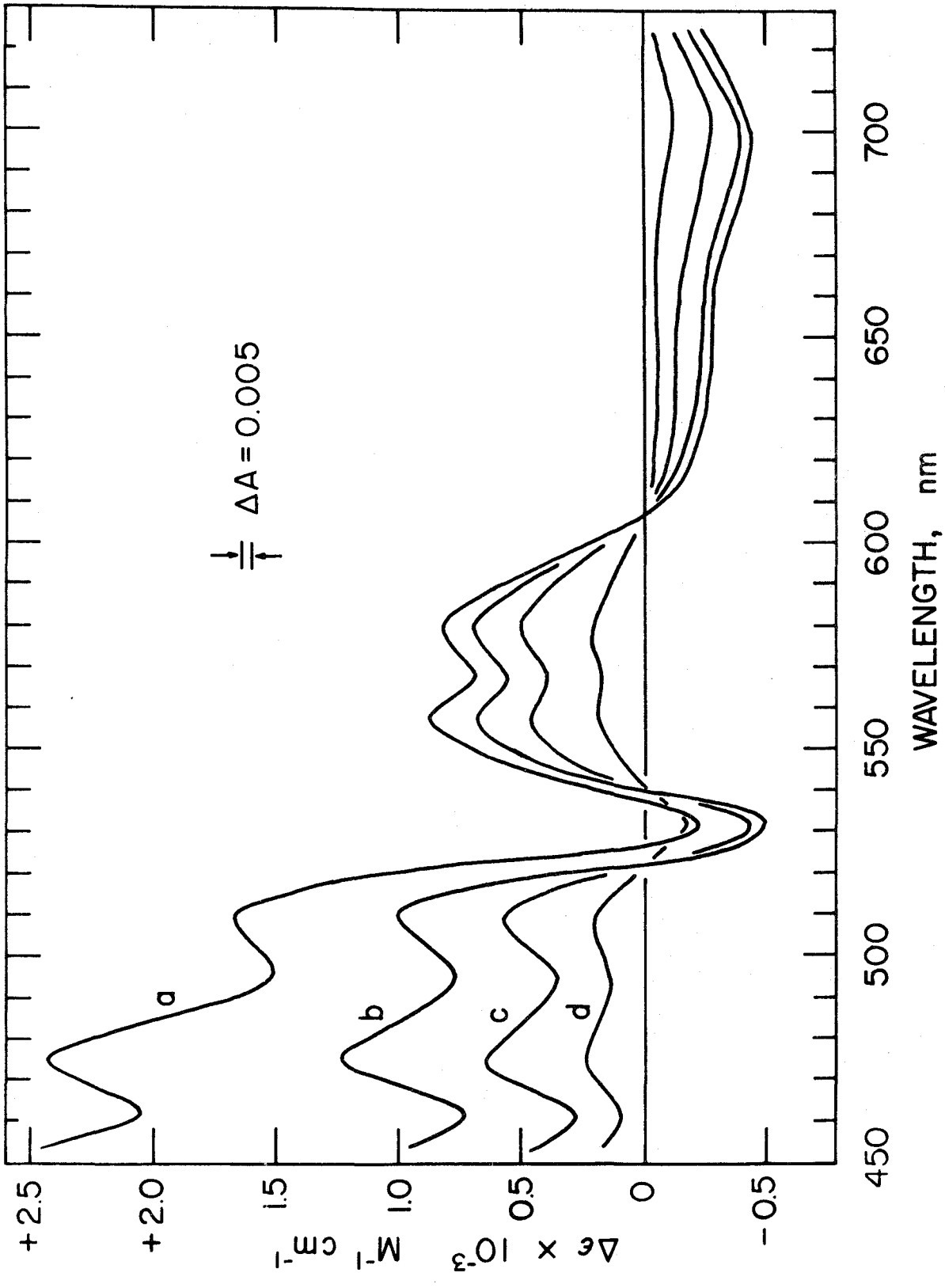
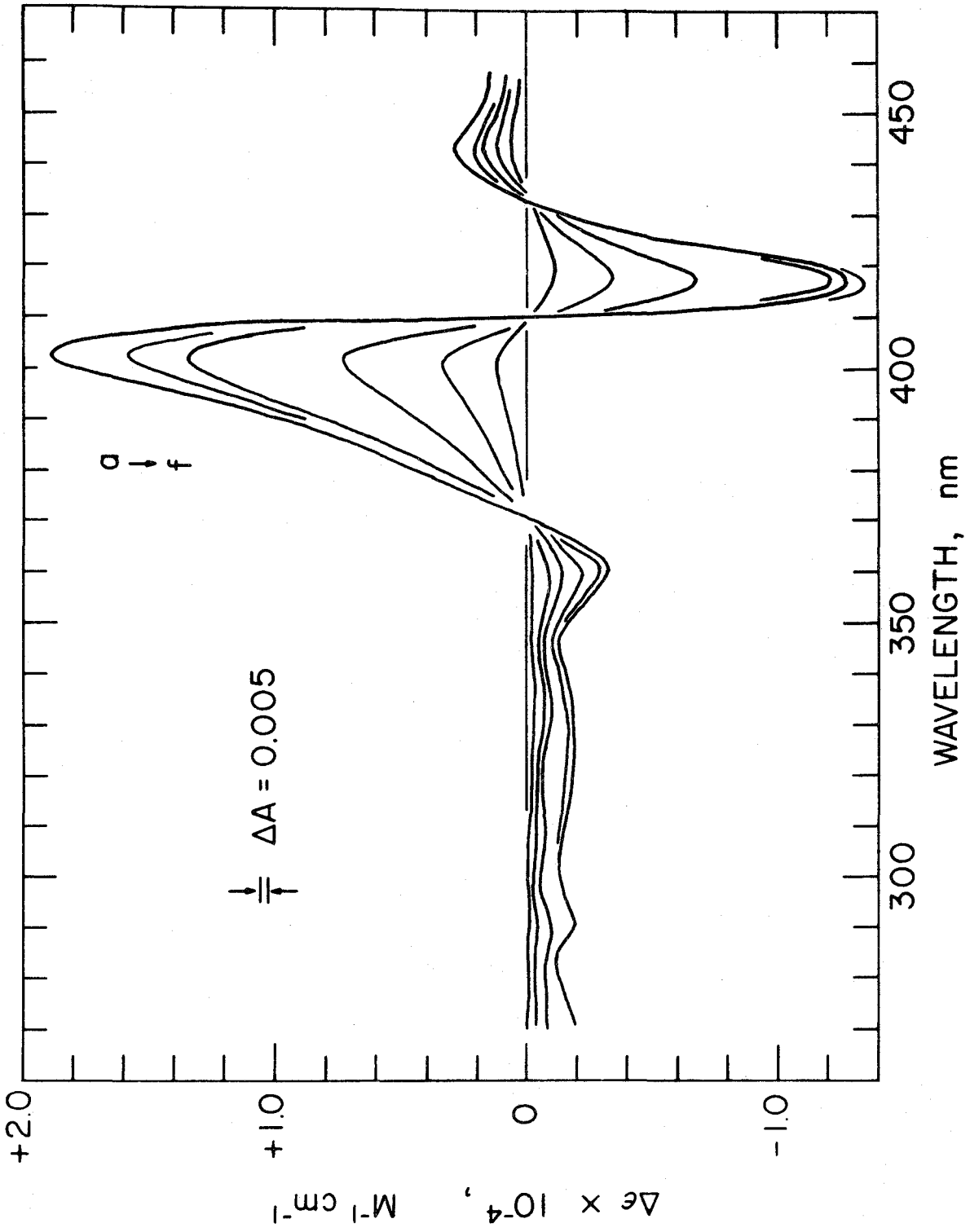

ΔA = 0.005
a
b
c
d
+2.5
+2.0
1.5
1.0
0.5
0
-0.5
Δε x 10⁻³ M⁻¹ cm⁻¹
450
500
550
600
650
700
WAVELENGTH, nm

FIGURE 6

Alkaline pH Titration of 1-TNP-cHo^{III} in the Region From 260 to 470 nm. The pH-induced difference spectra of 1-TNP-cHo^{III} from 270 to 460 nm in 0.009 M cacodylic acid-KOH buffer. The concentration was 1.6×10^{-5} M, and solutions contained 2×10^{-5} M potassium ferricyanide. Temperature 25°C, reference pH was 6.93, and the pH measured were: a, 10.53; b, 9.51; c, 9.01; d, 8.18; e, 7.74; and f, 7.38.


+2.0
+1.0
0
-1.0
Δε x 10⁻⁴, M⁻¹ cm⁻¹
300
350
400
450
WAVELENGTH, nm
ΔA = 0.005
a → f

at wavelengths shorter than 450 nm. The results are shown in Figure 6.

The difference spectra of unmodified ferricytochrome c are shown in Figures 7 and 8 for comparison.

There are two principal wavelength regions where the effect of pH on the spectrum of 1-TNP-cHo^{III} is different from the effect on unmodified ferricytochrome c. If we compare Figures 5 and 6 (1-TNP-cHo^{III}) with Figures 7 and 8 (cHo^{III}) in the region from 300 to 370 nm and from about 420 to 540 nm, the difference spectra for 1-TNP-cHo^{III} differ from those for cHo^{III} by the presence in the former of a negative band from about 300 to 370 nm, and a positive band from about 430 to 540 nm. This is quite similar to the change found upon the reduction of 1-TNP-cHo^{III} (Fig. 4), which is due to the reduction of the trinitrophenyl group attached to the lysine residue to the triaminophenyl group. The spectra for the pH titration of 1-TNP-cHo^{III} suggest, therefore, that the TNP group is autoreduced at alkaline pH, in spite of the presence of about 10^{-5} M potassium ferricyanide. In fact the potassium ferricyanide is probably not a strong enough oxidizing agent to reoxidize the reduced TNP groups, and its concentration is probably insufficient to prevent their reduction by the reducing equivalents generated in ferricytochrome solutions at alkaline pH (Brady and Flatmark, 1971). The mechanism of this so-called

FIGURE 7

Alkaline pH Titration of cHo^{III} in the Region from 450 to 750 nm. The pH induced difference spectra of cHo^{III} in 0.007 M tris-cacodylic acid buffer with 10^{-5} M potassium ferricyanide. Temperature 25°C. Cytochrome c concentration 1.2×10^{-4} M. The reference pH was 7.15 and the pH measured were: a, 7.65; b, 7.92; c, 8.46; d, 8.71; e, 9.00; f, 9.43; g, 9.70; h, 10.05, and i, 10.50 [reproduced from Morton, (to be published)].

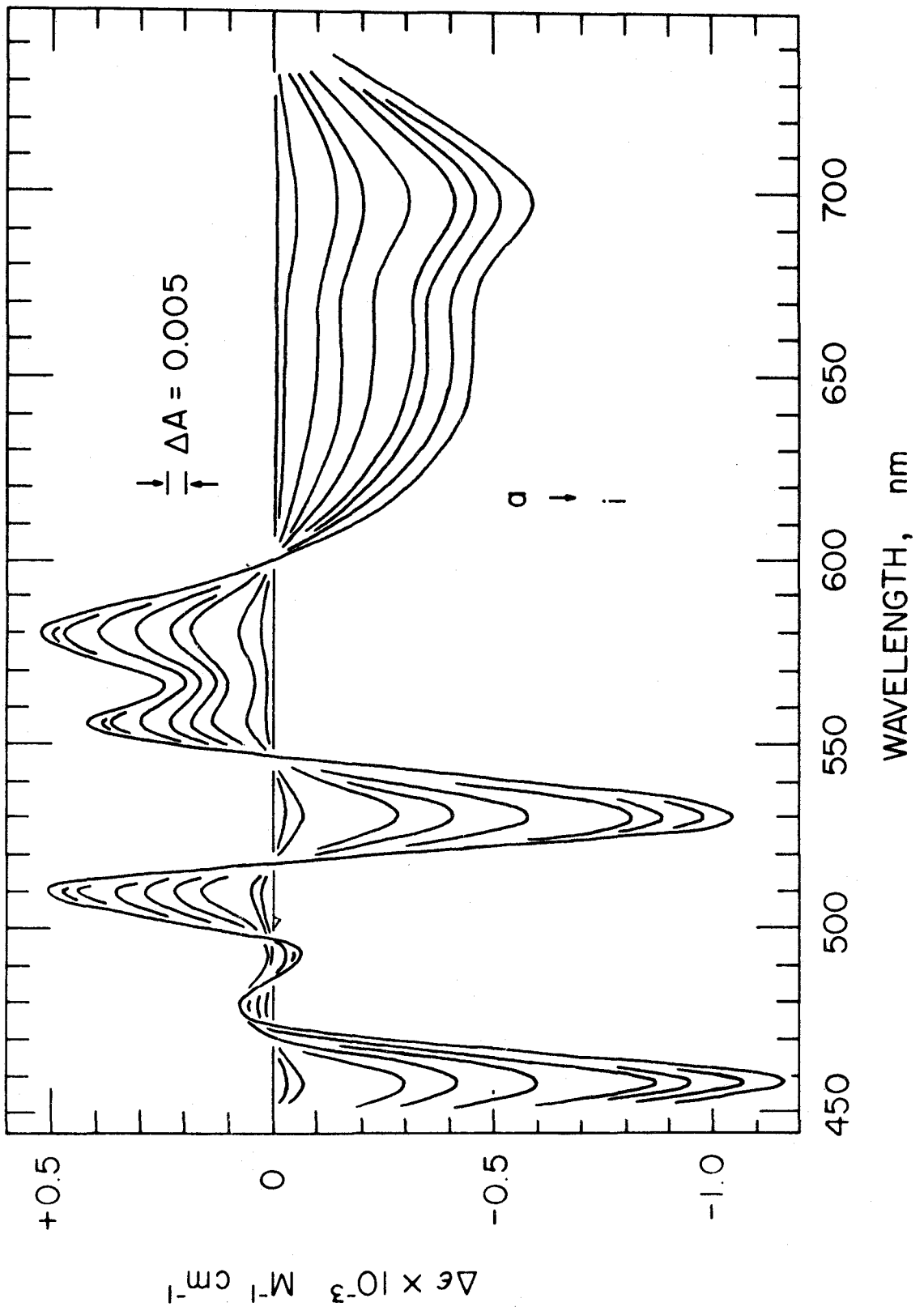
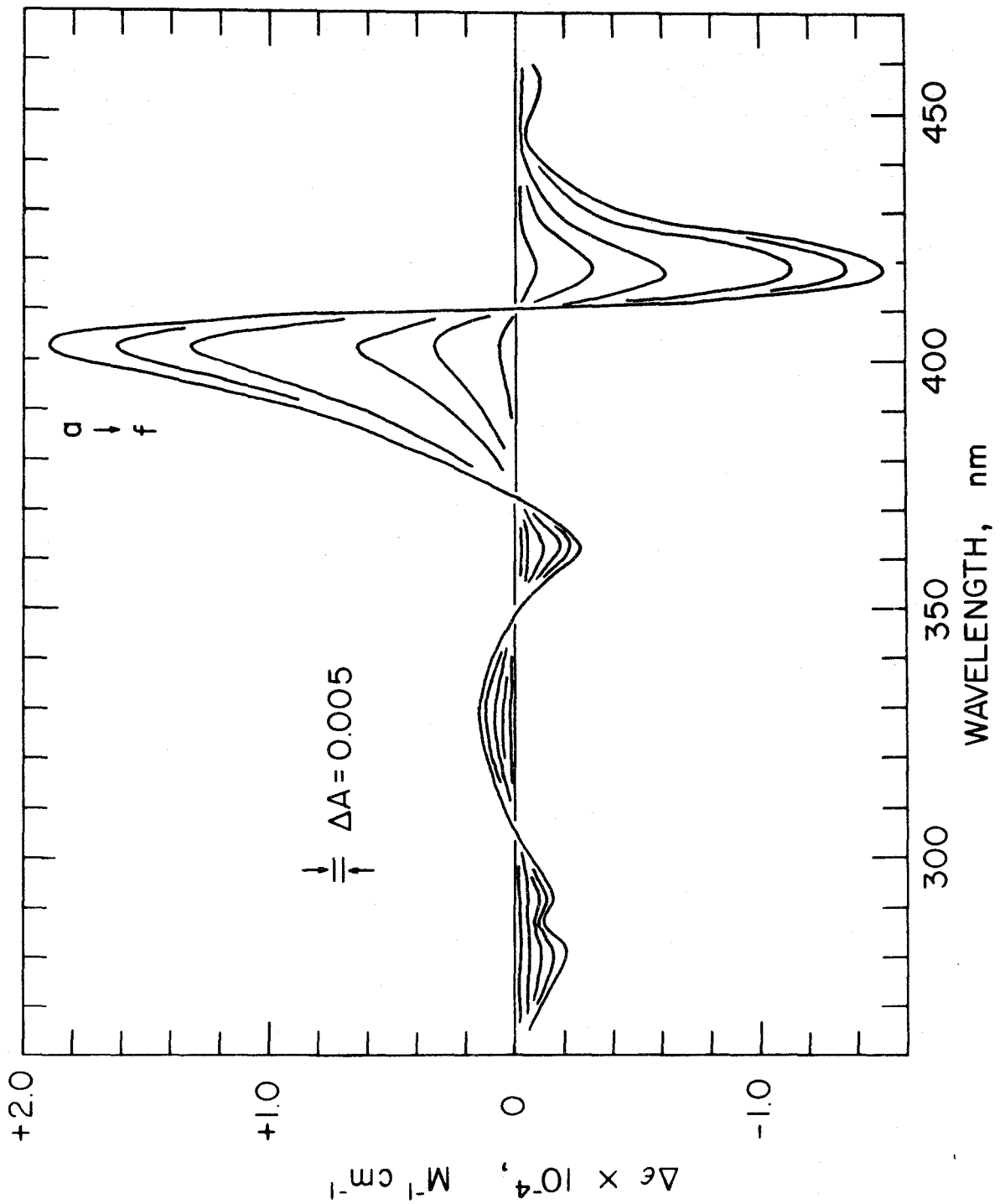

ΔA = 0.005
a → i
+0.5
0
-0.5
-1.0
Δε x 10⁻³ M⁻¹ cm⁻¹
450
500
550
600
650
700
WAVELENGTH, nm

FIGURE 8

Alkaline pH Titration of cHo^{III} in the Region from 260 to 470 nm. The pH-induced difference spectra of cHo^{III} in 0.01 M cacodylic acid-KOH buffer, with 10^{-5} potassium ferricyanide. Temperature 25°C. Concentration of protein was 1.6×10^{-5} M. The reference pH was 7.32, and the pH measured were: a, 10.32; b, 9.72; c, 9.14, d, 8.48; e, 8.12; and f, 7.68.


a → f
ΔA = 0.005
+2.0
+1.0
0
-1.0
Δε × 10⁻⁴, M⁻¹ cm⁻¹
300
350
400
450
WAVELENGTH, nm

autoreduction of ferricytochrome c at alkaline pH is so far unknown. These results, however, indicate that the TNP-lysine group at position 13 is an effective electron acceptor even in the absence of heme reduction.

The data of Figures 5 and 6 can be analyzed for the pK of the transition analogous to the type III to type IV transition in unmodified cytochrome c by the equation:

$$\log \frac{B}{A} = n\ pH - pK$$

for 1 proton ionization, n = 1, and B/A is calculated from:

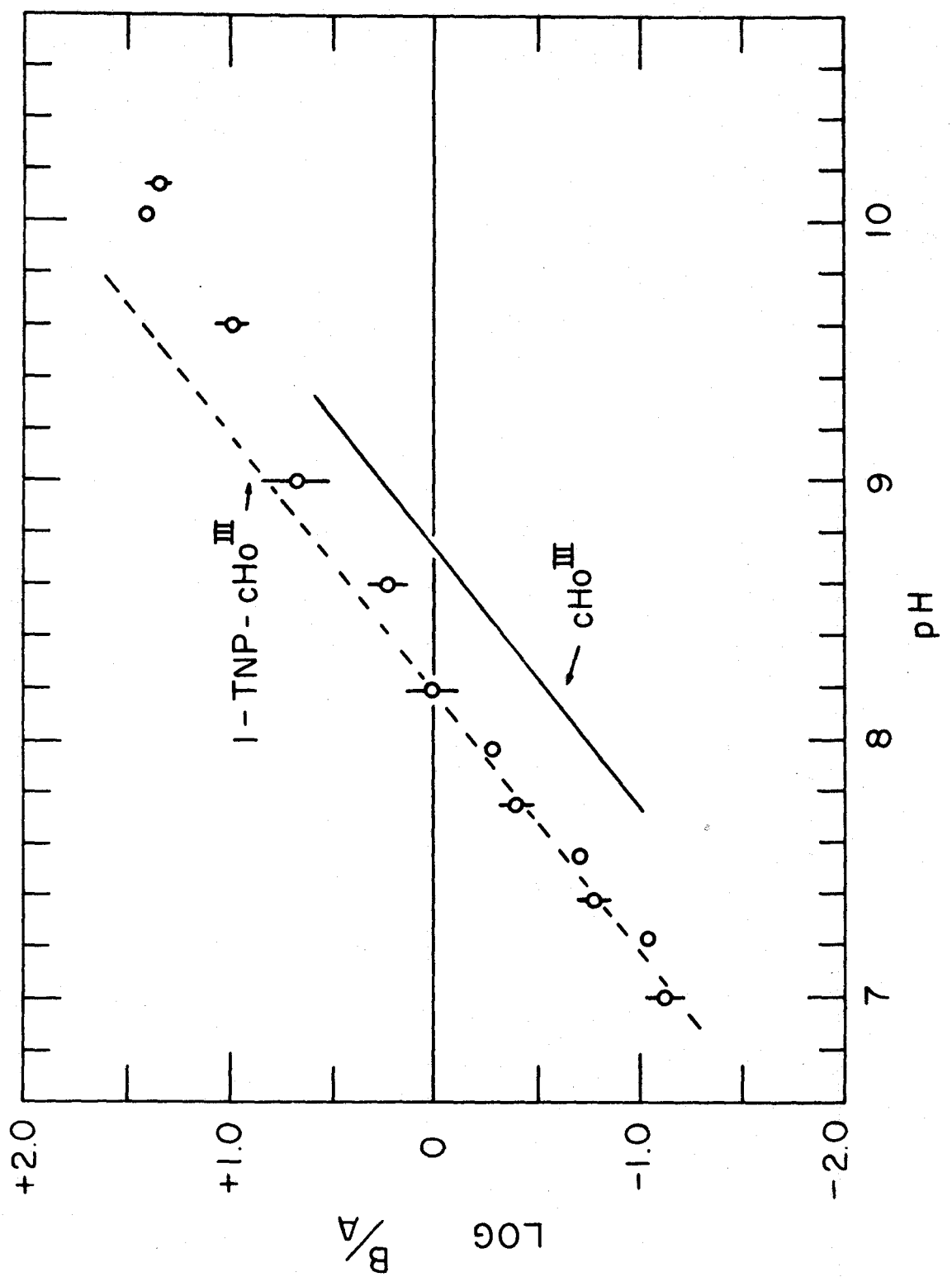
$$\frac{B}{A} = \frac{A_\lambda^{\,a} - A_\lambda}{A_\lambda - A_\lambda^{\,b}}$$

where $A_\lambda^{\,a}$ and $A_\lambda^{\,b}$ represent extrapolated absorbancies at low and high pH, respectively. A_λ represents the absorbancy at intermediate pH at different wavelengths.

This log B/A versus pH plot for 1-TNP-cHo^{III} is given in Figure 9 (and compared with the titration curve for unmodified protein). Titration curves from Figure 8 give the pK = 8.7. Morton (to be published) reported pK = 8.9 for unmodified cytochrome c. The pK of 1-TNP-cHo^{III} is shifted downwards about 0.6 units. This pK is even lower than that of fully modified Gn-cHo^{III} (pK = 9.4) and the TFA-cHo^{III}, pH = 10.3 (Morton, to be published).

FIGURE 9

The pK of pH-Induced Transitions for 1-TNP-cHo^{III} and cHo^{III}. Data from Figures 5 and 6 plotted according to the equation: $\log \frac{B}{A} = n\ pH - pK$ (see text). Absorbancies at the following wavelengths were used in the calculations: 403, 416, 644 and 700 nm. The solid line is the spectrometric titration curve determined under similar conditions for unmodified cytochrome c (data from Figure 8).


LOG B/A
+2.0
+1.0
0
-1.0
-2.0
7
8
9
10
pH
1-TNP-cHo III
cHo III

This suggests that the presence of the TNP group on lysine 13 disrupts to some degree the configuration of the active site, thus allowing the displacement of methionine 80 (perhaps by lysine 79) to occur at lower pK (lower OH^- concentration).

SECTION 2. Measurements of Equilibrium Constant in Reaction Between Ferricytochrome c and Ferrocyanide

Theory and Results

Whenever a molecule possesses two or more different functions, belonging to the nearby groups in the molecule, there is a possibility that these functions are inter-dependent. When this occurs, the functions are considered to be linked. In the case of cytochrome c, binding of ions and oxidation state are linked functions. The basic theory of linked functions was derived by Wyman (1964), and the same principles were used in the present analysis.

$$cHo^{II} + Fe(CN)_6^{3-} \rightleftharpoons cHo^{III} + Fe(CN)_6^{4-} \qquad (1)$$

In this reaction, not only cytochrome c forms complexes with ions in the solution, but also ferricyanide and ferrocyanide, which complicates the analysis. We must correct for this, but in many cases present data are not entirely satisfactory to allow the desired correction to be made. To make analysis possible, certain assumptions had to be made. These are:

1) Tris $[(CH_2OH)_3CNH_3^+]$ and cacodylate $[(CH_3)_2AsO_2^-]$ ions do not bind to either ferro- or ferricytochrome c.

2) Negative ions do not bind to either ferrocyanide or ferricyanide anions.

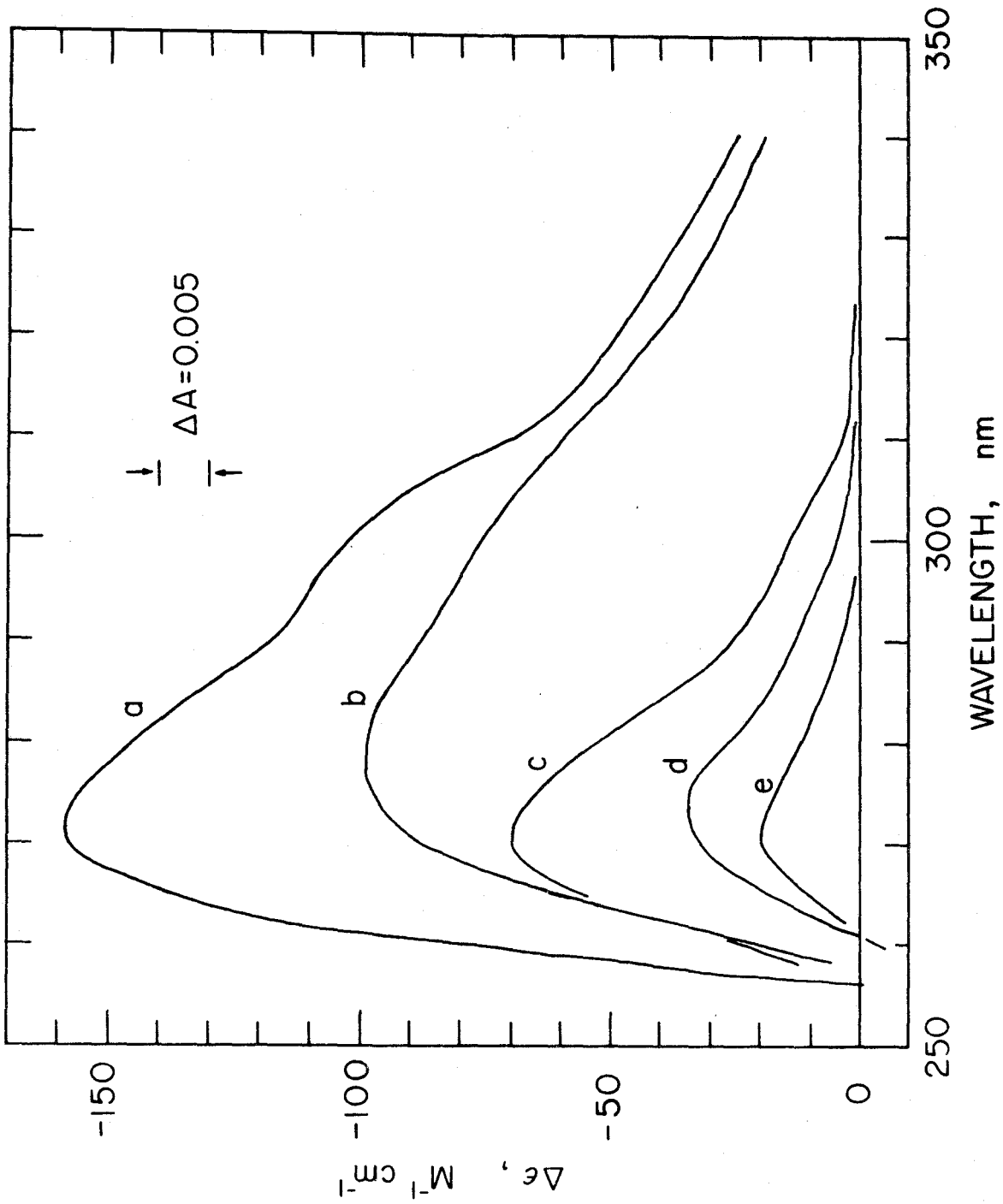
Analysis of data would be considerably simplified if we could assume that the tris cation did not bind to ferrocyanide or ferricyanide. Then the extensive data of Eaton et al. (1967) on binding of K^+ to ferrocyanide and ferricyanide would be sufficient to make the desired correction. However, spectrophotometric measurements show that tris does bind to ferrocyanide. Fig. 10 gives difference spectra of $K_4Fe(CN)_6$ in the presence of increasing concentrations of tris-HCl buffer at pH 7.0.

The difference spectra of Figure 10 are similar to those for the binding of other cations (K^+, Ba^{2+}, Mg^{2+}) to ferrocyanide (Cohen and Plane, 1957). Analysis of the data of Figure 10 indicates that association constant for tris cation and ferrocyanide is similar in magnitude to that for K^+ and ferrocyanide.

Spectrophotometric titration does not allow such a precise measurement of binding constants nor their variation with ionic strengths as does potentiometric titration. Furthermore, the spectrophotometric titration cannot measure the interaction of cations with ferricyanide, since K^+ and similar ions do not produce any perturbation of the ferricyanide absorption spectra, even though the potentiometric titrations show strong binding (Eaton et al.

FIGURE 10

Difference Spectra of Potassium Ferrocyanide Induced by Tris Binding. In the reference cuvet, the concentration of potassium ferrocyanide was 4.8×10^{-4} M, and the concentration of tris is zero. The samples measured had the same concentration of ferrocyanide, and the concentrations of tris were: a, 9.15×10^{-2} M; b, 4.72×10^{-2} M; c, 8.75×10^{-3} M; d, 4.59×10^{-3} M; e, 0.957×10^{-3} M. Temperature 25°C, pH 7.0.


ΔA=0.005
a
b
c
d
e
-150
-100
-50
0
250
300
350
WAVELENGTH, nm
Δε , M⁻¹ cm⁻¹

1967). Thus we have no available data on the interaction of tris with ferricyanide.

To summarize, the interpretation of equilibrium constant of reaction 1) is complicated by the reactions:

$$Fe(CN)_6^{3-} + C^+ \overset{K_2}{\rightleftharpoons} CFe(CN)_6^{2-} \qquad (2)$$

$$Fe(CN)_6^{4-} + C^+ \overset{K_3}{\rightleftharpoons} CFe(CN)_6^{3-} \qquad (3)$$

Where C^+ is any cation in solution. Furthermore the association constants (K_2 and K_3) are not necessarily the same, nor the same functions of ionic strength, nor are they known accurately for tris in reaction (3), and are completely unknown for tris in reaction (2).

A. Effect of ionic strength on reaction (1)

By the assumption that neither tris nor cacodylate ions bind to cytochrome c, the effect of varying the concentrations of these ions (at constant pH and temperature) on the apparent equilibrium constant of reaction (1) should be due to the combination of the effect of ionic strength on reaction (1) and cation binding on reactions (2) and (3). Unavoidably some K^+ ion is introduced into the reaction mixture from added potassium ferrocyanide. We shall ignore the binding of K^+ to cytochrome c, but correct for its binding to ferrocyanide and ferricyanide (reactions (2) and (3)).

In any case the concentration of K^+ in this series of experiments is essentially constant, and so apart from a constant change in the apparent equilibrium constant, the only uncorrected effect of K^+ will be the ionic strength dependence of its binding to cytochrome c.

The apparent equilibrium constant of reaction (1) is given by:

$$K_{app} = \frac{\Sigma(cHo^{III}) \quad \Sigma[Fe(CN)_6^{4-}]}{\Sigma[Fe(CN)_6^{3-}] \quad \Sigma(cHo^{II})} \qquad (A1)$$

where Σ refers to totality of all forms of ionic species in the solution. We have assumed that cytochrome c does not bind tris or cacodylate, and that we can ignore the effect of K^+ from the added ferrocyanide. Therefore:

$$K_{app} = \frac{(cHo^{III}) \quad \Sigma[Fe(CN)_6^{4-}]}{(cHo^{II}) \quad \Sigma[Fe(CN)_6^{3-}]} \qquad (A1')$$

The forms of ferro- and ferricyanide refer to free anions and their singly complexed forms with K^+ and tris cation (T^+). Multiple complexes of these two ions will be neglected.

$$Fe(CN)_6^{3-} + K^+ \underset{}{\overset{K_2}{\rightleftharpoons}} KFe(CN)_6^{2-} \qquad (A2)$$

$$Fe(CN)_6^{3-} + T^+ \underset{}{\overset{K_2'}{\rightleftharpoons}} TFe(CN)_6^{2-} \qquad (A2')$$

$$Fe(CN)_6^{4-} + K^+ \underset{}{\overset{K_3}{\rightleftharpoons}} KFe(CN)_6^{3-} \qquad (A3)$$

$$Fe(CN)_6^{4-} + T^+ \underset{}{\overset{K_3'}{\rightleftharpoons}} TFe(CN)_6^{3-} \qquad (A3')$$

From the above four equations:

$$[KFe(CN)_6^{2-}] = K_2 \ (K^+) \ [Fe(CN)_6^{3-}]$$

$$[TFe(CN)_6^{2-}] = K_2' \ (T^+) \ [Fe(CN)_6^{3-}]$$

$$[KFe(CN)_6^{3-}] = K_3 \ (K^+) \ [Fe(CN)_6^{4-}]$$

$$[TFe(CN)_6^{3-}] = K_3' \ (T^+) \ [Fe(CN)_6^{4-}]$$

$$\Sigma[Fe(CN)_6^{4-}] = [Fe(CN)_6^{4-}] \ [1 + K_3(K^+) + K_3'(T^+)]$$

$$\Sigma[Fe(CN)_6^{3-}] = [Fe(CN)_6^{3-}] \ [1 + K_2(K^+) + K_2'(T^+)]$$

$$K_{app} = \frac{(cHo^{III}) \ [Fe(CN)_6^{4-}]}{(cHo^{II}) \ [Fe(CN)_6^{3-}]} \ \frac{[1 + K_3(K^+) + K_3'(T^+)]}{[1 + K_2(K^+) + K_2'(T^+)]} \qquad (A4a)$$

Let: $$F(I) = \frac{[1 + K_3(K^+) + K_3'(T^+)]}{[1 + K_2(K^+) + K_2'(T^+)]}$$

and $$K = \frac{(cHo^{III}) \ [Fe(CN)_6^{4-}]}{(cHo^{II}) \ [Fe(CN)_6^{3-}]}$$

then:

$$K_{app} = K \quad F(I) \tag{A4b}$$

We will use data from Eaton et al. (1967) for apparent association constants. These are:

$$\log K_i = \log K_i^{o} - \frac{n \sqrt{I}}{1 + 1.5 \sqrt{I}} \tag{A5}$$

for K_3, $\log K_3^{o} = 2.35$, $n = 4.08$

K_2, $\log K_2^{o} = 1.46$, $n = 3.06$

K_3' and K_2' are unknown, but K_3' is the same order as K_3. In equation A4b, K is the equilibrium constant which would be measured in absence of ion association of ferrocyanide and ferricyanide. This equilibrium constant is ionic strength dependent.

$$K = \frac{(cHo^{III}) \quad [Fe(CN)_6^{4-}]}{(cHo^{II}) \quad [Fe(CN)_6^{3-}]} \tag{A6}$$

The chemical potentials, based on single ion activity coefficients for the four ions are:

$$\mu_{cHo^{II}} = \mu^{o}_{cHo^{II}} + 2.3 \; RT \log [\gamma_{cHo^{II}} \; (cHo^{II})]$$

$$\mu_{cHo^{III}} = \mu^{o}_{cHo^{III}} + 2.3 \; RT \log [\gamma_{cHo^{III}} \; (cHo^{III})]$$

$$\mu_{Fe(CN)_6^{4-}} = \mu^o_{Fe(CN)_6^{4-}} + 2.3\ RT\ \log\ (\gamma_{Fe(CN)_6^{4-}}\ [Fe(CN)_6^{4-}])$$

$$\mu_{Fe(CN)_6^{3-}} = \mu^o_{Fe(CN)_6^{3-}} + 2.3\ RT\ \log\ (\gamma_{Fe(CN)_6^{3-}}\ [Fe(CN)_6^{3-}])$$

At equilibrium, $\Sigma\ \mu_i = 0$.

We define:

$$2.3\ RT\ \log K^o = \mu^o_{cHo^{II}} + \mu^o_{Fe(CN)_6^{3-}} - \mu^o_{cHo^{III}} - \mu^o_{Fe(CN)_6^{4-}}$$

Therefore:

$$0 = -2.3\ RT\ \log K^o + 2.3\ RT\ \log \frac{(cHo^{III})\ [Fe(CN)_6^{4-}]}{(cHo^{II})\ [Fe(CN)_6^{3-}]}$$

$$+ 2.3\ RT\ \log \frac{\gamma_{cHo^{III}}\ \gamma_{Fe(CN)_6^{4-}}}{\gamma_{cHo^{II}}\ \gamma_{Fe(CN)_6^{3-}}}$$

We define:

$$\Delta\gamma = \frac{\gamma_{cHo^{III}}\ \gamma_{Fe(CN)_6^{4-}}}{\gamma_{cHo^{II}}\ \gamma_{Fe(CN)_6^{3-}}}$$

From the above, we derive equation (A7):

$$K = K^o\ (\frac{1}{\Delta\gamma}) \qquad \text{(A7)}$$

Equation (A7) combined with equation (A4) gives:

$$K_{app} = \left(\frac{F(I)}{\Delta\gamma}\right) K^{o} \qquad (A8)$$

where $F(I)$ and $\Delta\gamma$ are ionic strength dependent, and $\frac{F(I)}{\Delta\gamma} \rightarrow 1$ as $I \rightarrow 0$. We use the Debye and Hückel Theory for activity coefficients of ions of a finite size (Tanford, 1961).

$$-\log\gamma_z = \frac{z^2 \; A\sqrt{I}}{1 + Bd\sqrt{I}} \qquad (A9)$$

Where A and B are constants. At T = 25°C in aqueous solution, $A = 0.509\ (\text{mole/L})^{-1/2}$ and $B = 0.329 \times 10^{8}\ (\text{mole/L})^{-1/2}\ \text{cm}^{-1}$ (Paul, 1951), and d is the distance of closest approach between the fixed ion and its mobile sphere of counter ions. Estimation of the closest approach of ions to cytochrome c gives $d_{cHo} \approx 20$ Å, and $d_{\text{ferro/ferri-cyanide}} \approx 8$Å.

From the definition of $\Delta\gamma$:

$$-\log\Delta\gamma = \log(\gamma_{cHo^{III}}) + \log(\gamma_{Fe(CN)_6^{4-}}) - \log(\gamma_{cHo^{II}}) - \log(\gamma_{Fe(CN)_6^{3-}}) \qquad (A10)$$

By using the appropriate values of d, given above, the equation (A10) and (A9) together give:

$$- \log \Delta\gamma = \frac{7\ A\sqrt{I}}{1 + 2.6\sqrt{I}} + \frac{(2z_{II} + 1)\ A\sqrt{I}}{1 + 6.6\sqrt{I}} \qquad \text{(A11)}$$

where z_{II} represents net charge of cHo^{II} and $z_{III} = z_{II} + 1$. The purpose of allowing the charge on the cytochrome c molecule to be variable is to take into account the possible presence of deaminated forms having more negative charge than the native molecule (Flatmark, 1966).

The results are plotted according to the equation A 12 as $\log K_{app}$ versus $- \log \Delta\gamma$.

$$\log K_{app} = \log K^{o} + \log F(I) - \log \Delta\gamma \qquad \text{(A12)}$$

The intercept at $I = 0$ gives $\log K^{o}$ which is related to the standard reduction potential of cytochrome c at pH 7.0.

We list the following uncertainties in plotting the apparent equilibrium constant according to the equation (A12):

1) Uncertainties in the net charge on the cytochrome c molecule (due to the deaminated forms).
2) Uncertainties in calculating F(I) due to the binding of tris to ferrocyanide and ferricyanide.
3) Approximations involved in the use of the single ion activity coefficient (equation A9) in a solution of mixed electrolytes at finite ionic strength.

In order to test equation A12 we plot the data for unmodified cytochrome c using various assumptions about the binding of tris. The ionic strength is varied by changing the concentration of tris-cacodylate buffer, while the amount of potassium ferrocyanide added is approximately constant. The concentration of free potassium ion was estimated according to equation A13. We assumed that the amount of ferrocyanide reacting to give ferricyanide was negligible. The initial concentration of ferrocyanide was the order of magnitude 1.5×10^{-4} M, and tris-cacodylate of the order of 3 to 8×10^{-3} M.

$$(K^+) \approx (K^+)_o - [KFe(CN)_6^{3-}] \qquad (A13)$$

The concentration of free tris cation was estimated according to equation (A14):

$$(T^+) \approx \frac{(T)_o}{1 + K_+(H^+)} \qquad (A14)$$

where K_+ is ionization constant, $K_+ = 10^{-8.02}$ and $(T)_o$ is the added tris concentration.

The correction due to cation binding [F(I)] was calculated for four different assumptions [see equation A4a) for the definition of F(I)]. In all cases K_3 and K_2 were obtained from data of Eaton *et al.* (1967) by

equation (A5). K_3' and K_2' are the association constants for the tris cation with ferrocyanide and ferricyanide respectively (equations A2' and A3'). The various assumptions were:

A: $K_3' = K_2' = 0$ (no tris binding).

B: $K_3' = K_3$; $K_2' = K_2$ (tris binding similar to potassium binding).

C: $K_3' = K_3$; $K_2' = 0$ (tris does not bind to ferricyanide).

D: $K_3' = K_3$; $K_2' = K_3$ (tris binds to ferrocyanide and ferricyanide equally well).

Figure 11 gives the plot for cytochrome c according to equation A12. The charge on the reduced species is assumed to be 9.5 ± 0.5(Margaliosh and Schejter, 1966). The errors in the apparent equilibrium constant are standard errors in a series of five measurements.

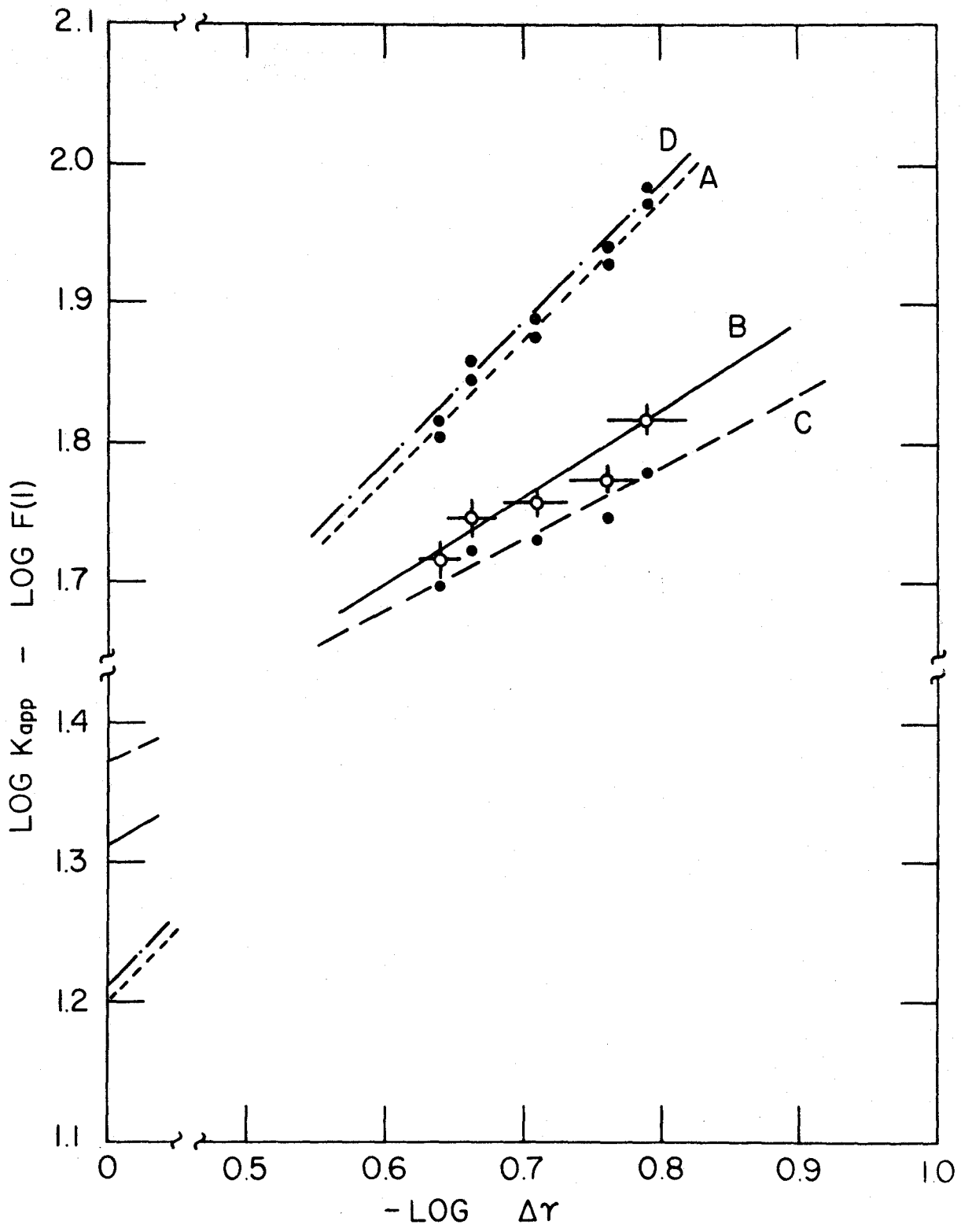
The result of Figure 11 shows that both assumptions B and C in which tris strongly discriminates between ferrocyanide and ferricyanide give a slope significantly different from 1.0 and intercept the order of 1.35 ± 0.05 while assumptions A and D give a slope of approximately 1, and an intercept at about 1.2 ± 0.05.

The value of log K^o is related to the standard reduction potential at pH 7.0 and 25°C of the cytochrome c and ferri/ferrocyanide couple by the equation:

$$\log_{10} K^o = \frac{1}{0.0592} [E_{o_1}' - E_{o_2}'] \qquad \text{(A15)}$$

FIGURE 11

The Effect of Ionic Strength on the Apparent Equilibrium Constant Taking into Account Different Assumptions About Ion Association Constants. Data for the apparent equilibrium constant for the reaction of ferrocyanide and ferricytochrome c plotted according to equation A12; $-\log \Delta\gamma$ is related to the ionic strength. Corrections F(I) were calculated according to four different assumptions: A: $K_3' = K_2' = 0$; B: $K_3' = K_3$, $K_2' = K_2$; C: $K_3' = K_3$, $K_2' = 0$; D: $K_3' = K_2' = K_3$; using equation A4a. The ionic strengths varied from 3.9×10^{-3} M to 7.1×10^{-3} M. The cytochrome c concentration is in the range from 0.9 to 1.2×10^{-5} M. The concentration of potassium ferrocyanide is 1.6×10^{-4} M. The errors on curve B are for the standard errors of the measured K_{app} values, and a variation in the charge of ferrocytochrome c of ±0.5 units. Temperature 25°C, pH 7.0.


2.1
2.0
1.9
1.8
1.7
1.4
1.3
1.2
1.1
LOG Kapp - LOG F(1)
D
A
B
C
0
0.5
0.6
0.7
0.8
0.9
1.0
- LOG Δγ

Where E_{o_1}' is standard reduction potential of ferri-ferrocyanide couple at zero ionic strength. This value is 0.355 V (Hanania et al., 1967). E_{o_2}' is standard reduction potential of cytochrome c at zero ionic strength, and 0.0592 [V] = 2.303 $\frac{RT}{F}$ for 25°C. Unfortunately the standard reduction potential of cytochrome c at zero ionic strength is not known. Schejter and Margalit (1970) give a value of 0.274 V from measurements similar to these, but they have apparently ignored the possibility of tris binding (case A in Fig. 11). Frohwirth (1962) reports a value of 0.238 V for the ionic strength about 0.1 M, measured by two different methods, but there is no report on E_o' for ionic strength zero.

The intersection of 1.35 corresponds to E_{o_2}' = 0.275 V while that of 1.2 corresponds to E_{o_2}' = 0.284 V. The former value of E_{o_2}' is about the same as found by Schejter and Margalit (1970) but the slope is only about 0.6. We shall assume that the tris cation does not distinguish strongly between ferrocyanide and ferricyanide and assumption D is most reasonably applied to the experimental data.

B. The effect of ionic strength on reaction (1) using cytochrome c with modified lysine residues

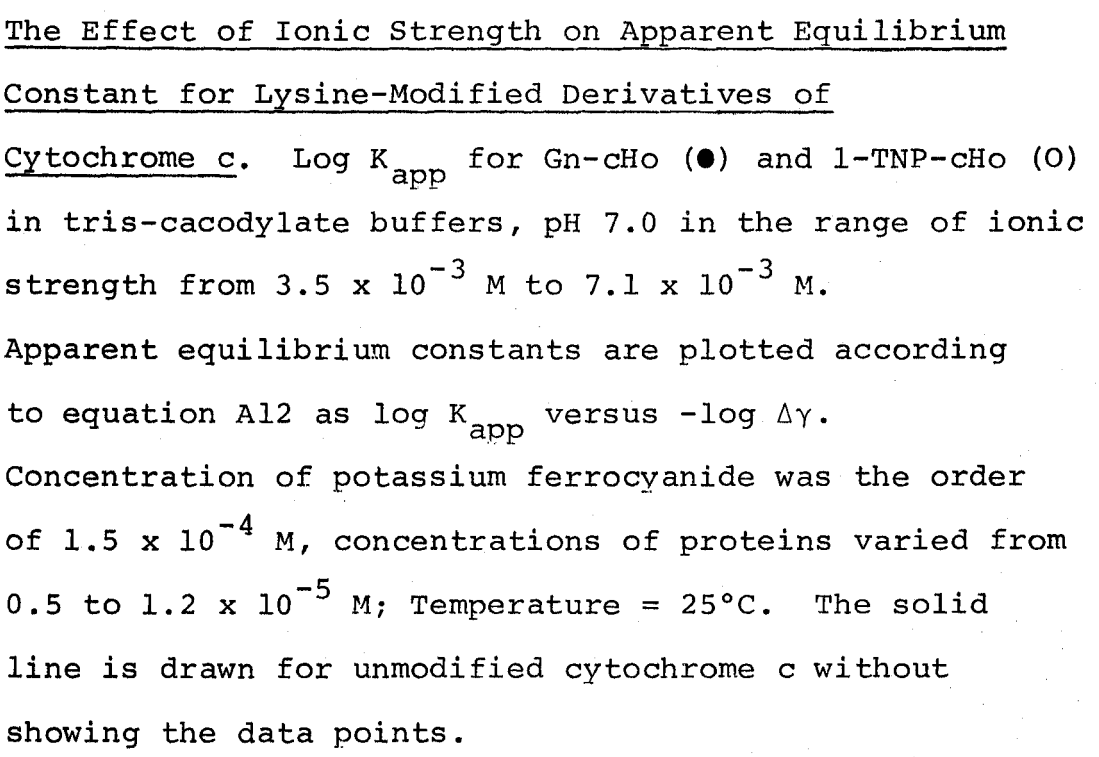
Whatever the correction F(I) for cation binding to ferrocyanide and ferricyanide it is independent of the nature of cytochrome c used. If we make the assumption that tris binds to ferrocyanide and ferricyanide about equally well, the correction [F(I)] is nearly negligible compared to the error involved in reproducing repeated measurements. Figure 12 gives log K_{app} (without the correction) versus -log $\Delta\gamma$ for the series of modified proteins. The values of $\Delta\gamma$ (dependent on ionic strength) were calculated on the basis of the following assumptions about the net charge of the reduced species.

cHo^{II}	$z_{II} = +9.5$
$Gn\text{-}cHo^{II}$	$z_{II} = +9.5$
$1\text{-}TNP\text{-}cHo^{II}$	$z_{II} = +8.5$

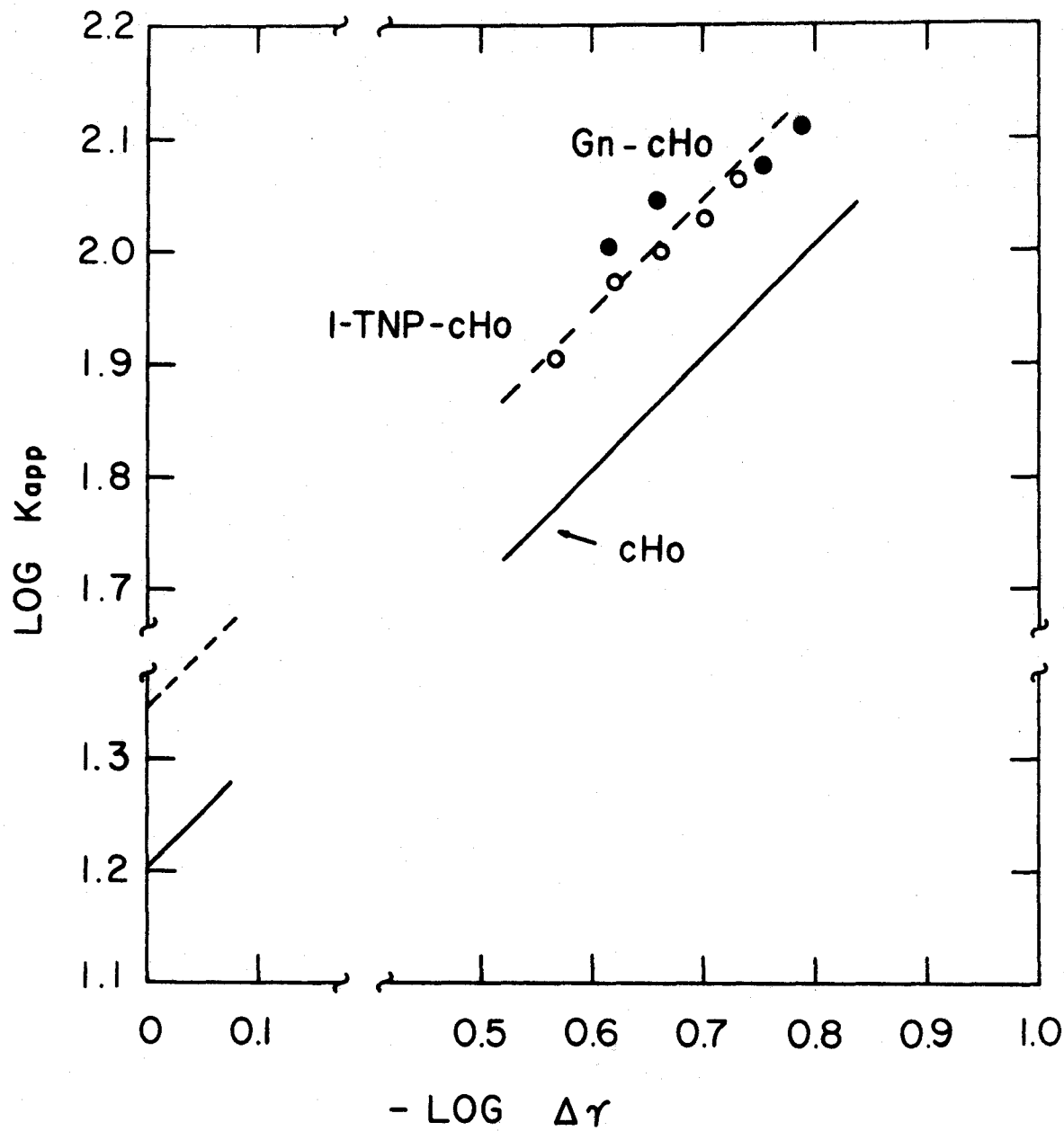
For guanidinated cytochrome c the net charge is the same as for unmodified, while in the case of $1\text{-}TNP\text{-}cHo^{II}$, one lysine is assumed to have lost its positive charge.

In the case of Gn-cHo, the slope seems to be somewhat less than 1.0. However, we have evidence that this derivative is extremely liable to aggregation upon storage. (This was at 4°C in 3×10^{-3} M tris-cacodylate buffer, pH 7.0.) Aggregation caused an increase in

FIGURE 12



The Effect of Ionic Strength on Apparent Equilibrium Constant for Lysine-Modified Derivatives of Cytochrome c. Log K_{app} for Gn-cHo (●) and 1-TNP-cHo (O) in tris-cacodylate buffers, pH 7.0 in the range of ionic strength from 3.5×10^{-3} M to 7.1×10^{-3} M. Apparent equilibrium constants are plotted according to equation A12 as log K_{app} versus -log $\Delta\gamma$. Concentration of potassium ferrocyanide was the order of 1.5×10^{-4} M, concentrations of proteins varied from 0.5 to 1.2×10^{-5} M; Temperature = 25°C. The solid line is drawn for unmodified cytochrome c without showing the data points.


2.2
2.1
2.0
1.9
1.8
1.7
1.3
1.2
1.1
LOG Kapp
Gn - cHo
I-TNP-cHo
cHo
0
0.1
0.5
0.6
0.7
0.8
0.9
1.0
- LOG Δr

apparent equilibrium constant. Measurements of equilibrium constants for Gn-cHo had to be made on relatively freshly prepared samples (up to about 1 month). Significant differences in apparent equilbrium constant were observed upon storage, for example K_{app} ($I = 5 \times 10^{-3}$) about 113 would increase to about 150 in a few weeks of storage. Therefore the most reasonable assumption is that the measurements for Gn-cHo at low ionic strengths in Figure 12 are somewhat higher than they should be, because of the presence of aggregated, denatured molecules. If this is the case, the reduction potential of Gn-cHo is essentially the same as for 1-TNP-cHo.

The intersections from Figure 12 can be converted to standard reduction potentials at pH 7.0 and zero ionic strength by using equation A15. The resulting values are given in Table 3.

In the case of TFA-cytochrome c, we were unable to obtain any quantitative data for E_o'. The modification which changes the net charge of the molecule from +9.5 to -9.5 in the reduced form causes significant changes in reduction potential. The differences between the reduction potential ferri/ferrocyanide couple and TFA-cHo are much larger than in the other types of modified proteins, which significantly increased the systematic error of measurements. Approximate estimate for E_o' for TFA-cHo is less than 0.1 V.

TABLE 3

Reduction Potentials of Cytochrome c and the Lysine-Modified Derivatives Extrapolated to Zero Ionic Strength

Cytochrome c Derivative	E_o' (Volts)
cHo	0.284*
Gn-cHo	0.275
1-TNP-cHo	0.275
TFA-cHo	<0.1

*Values in Table 3 are uncorrected for cation binding. Correction for cation binding according to the assumption D (page 48) brings this value up by about 0.002 volts.

To summarize the data:

1. Unmodified cytochrome c has a reduction potential at ionic strength zero about 0.284 ± 0.003 V. The result is reported uncorrected for F(I). This result is within the experimental error the same as the one corrected for F(I) calculated for assumption D (which seems the most probable).
2. Upon modification of lysine residues, the ionic strength behaviour of K_{app} does not change if the charge is not significantly altered.
3. The reduction potentials at zero ionic strength of Gn-cHo and 1-TNP-cHo are nearly the same as for unmodified cytochrome c.

4. Upon the modification with S-ethyltrifluoroacetate, where the net charge of reduced TFA cytochrome becomes -9.5 (assuming that all lysines lost their positive charge), the reduction potential of the protein is lowered to less than 0.1 volts. The value is estimated, because by the method employed we could not obtain more accurate data.

C. Binding of Cations to Cytochrome c

(a) The equilibrium constant for the system in the presence of binding ions is defined by:

$$K_{app} = \frac{\sum_i (cHo^{III})_i \; [Fe(CN)_6^{4-}]}{\sum_i (cHo^{II})_i \; [Fe(CN)_6^{3-}]} \; X \; F(I) \qquad (C1)$$

where $$F(I) = \frac{1 + K_3(K^+) + K_3'(T^+)}{1 + K_2(K^+) + K_2'(T^+)}$$

if we assume that the equilibrium of ferro- and ferricyanide can be completely described by equations A2, A2', A3 and A3'. The analysis of section A suggests that log F(I) is approximately constant over the range of ionic strengths used in these experiments.

Following Wyman (1964), we define a series of equilibrium constants for the interaction of ferro- and ferricytochrome with cations like K^+ and Na^+. The experimental arrangement is to keep the negative ion (cacodylate) constant, ionic strength and pH constant, and replace $tris^+$ by K^+ (or Na^+). The system of species at equilibrium is described by a series of equations:

for ferrocytochrome c:

$$cHo^{II} + c^+ \overset{K_{a_1}}{\rightleftharpoons} c\ cHo^{II} \qquad (Ca1)$$

$$cHo^{II} + 2c^{+} \overset{K_{a_2}}{\rightleftharpoons} c_2\ cHo^{II} \qquad (Ca2)$$

:

$$cHo^{II} + nc^{+} \overset{K_{a_n}}{\rightleftharpoons} c_n\ cHo^{II} \qquad (Can)$$

and for ferricytochrome c:

$$cHo^{III} + c^{+} \overset{K_{b_1}}{\rightleftharpoons} c\ cHo^{III} \qquad (Cb1)$$

:

$$cHo^{III} + n'c^{+} \overset{K_{b_{n'}}}{\rightleftharpoons} C_{n'}\ cHo^{III} \qquad (Cbn')$$

The association constants describe the following ratios of concentrations at equilibrium.

$$K_{a_i} = \frac{(c_i\ cHo^{II})}{(cHo^{II})(c^{+})^{i}}$$

$$K_{b_i} = \frac{(c_i\ cHo^{III})}{(cHo^{III})(c^{+})^{i}}$$

Therefore:

$$\sum_i (cHo^{II})_i = (cHo^{II}) \left[\sum_{i=0}^{n} K_{a_i} (c^{+})^{i}\right] \qquad (C2)$$

$$\sum_i (cHo^{III})_i = (cHo^{III}) \left[\sum_{i=0}^{n'} K_{b_i} (c^{+})^{i}\right] \qquad (C3)$$

$$K_{a_o} = K_{b_o} = 1$$

n and n' represent the total number of cation binding sites in ferrocytochrome and ferricytochrome c, respectively. Combining equations C1, C2 and C 3 together:

$$K_{app} = \frac{(cHo^{III})[Fe(CN)_6^{4-}]}{(cHo^{II})[Fe(CN)_6^{3-}]} \times F(I) \times \frac{\sum_{i=0}^{n'} K_{b_i} (c^+)^i}{\sum_{i=0}^{n} K_{a_i} (c^+)^i}$$

Using equations A6 and A7:

$$K_{app} = K\, F(I) \quad \frac{\sum_{i=0}^{n'} K_{b_i} (c^+)^i}{\sum_{i=0}^{n} K_{a_i} (c^+)^i} \qquad (C4)$$

The difficulty in analysis of the data is that even though the ionic strength is held constant, F(I) need not be constant since c^+ and T^+ [i.e., K^+ or Na^+ and T^+ (the tris cation)] vary inversely. There is always an uncertainty as to whether the observed variation in K_{app} is due to differential cation binding of tris to ferrocyanide or to cytochrome c The lack of exact knowledge about F(I) means that we cannot assign the measured variation in K_{app} with c^+ to the first factor in C4, or to the second.

Following Wyman:

$(X)_{red}$=total concentration of bound ligand to ferrocytochrome c.

$(X)_{ox}$=total concentration of bound ligand to ferricytochrome c.

$$(X)_{red} = (cHo^{II}) \sum_{i=0}^{n} iK_{a_i} (c^+)^i$$

$$(X)_{ox} = (cHo^{III}) \sum_{i=0}^{n'} iK_{b_i} (c^+)^i$$

The total concentrations of ferro and ferricytochrome c are given by C2 and C3. Therefore we can write the average number of moles of cation bound per molecule of ferro- and ferricytochrome c as:

$$\bar{X}_{red} = \frac{\sum_{i=0}^{n} iK_{a_i} (c^+)^i}{\sum_{n=0}^{n} K_{a_i} (c^+)^i} \qquad (C5)$$

$$\bar{X}_{ox} = \frac{\sum_{i=0}^{n'} iK_{b_i} (c^+)^i}{\sum_{i=0}^{n'} K_{b_i} (c^+)^i} \qquad (C6)$$

This is just:

$$\bar{X}_{red} = \frac{d[\log \sum_{i=0}^{n} K_{a_i} (c^+)^i]}{d \log (c^+)} \qquad (C7)$$

$$\bar{X}_{ox} = \frac{d[\log \sum_{i=0}^{n'} K_{b_i} (c^+)^i]}{d \log (c^+)} \qquad (C8)$$

Therefore combining C4 with C7 and C8 we plot log $[\frac{K_{app}}{F(I)}]$ versus log (c^+), the slope at any point is $\bar{X}_{ox}-\bar{X}_{red}$, the difference in average number of moles of cation bound to oxidized and reduced form of cytochrome c.

In order to test equation C4, we plot the data for unmodified cytochrome c at one ionic strength, using various assumptions about the binding of tris. F(I) was calculated according to equation A4a, and free potassium and tris concentrations were calculated from equations A13 and A14.

The four assumptions were:

A: $K_3' = K_2' = 0$ (no tris binding)

B: $K_3 = K_3'$, $K_2 = K_2'$ (tris binding similar to potassium binding).

C: $K_3 = K_3'$, $K_2' = 0$ (tris does not bind to ferricyanide).

D: $K_3' = K_3$, $K_2' = K_3$ (tris binds ferrocyanide and ferricyanide equally well).

These assumptions are the same as in Section 2B. Figure 13 gives log $[K_{app}/F(I)]$ versus log (K^+) for unmodified cytochrome c.

If we compare the calculated average slopes for the different assumptions with that obtained for uncorrected values of K_{app}, we see that assumptions C and B do not change the slope at all. The changes are observed in the cases A and D, the most reasonable one being assumption D. The slope in Figure 13 represents $\bar{X}_{ox} - \bar{X}_{red}$, the difference in average number of moles of potassium ion bound to the two redox forms of cytochrome c.

FIGURE 13

Effect of Correction for Cation Association on Potassium Binding to Cytochrome c. The variation of the apparent equilibrium constant corrected according to equation C4 versus free K^+ concentration. F(I) was calculated for four different assumptions: A: $K_3' = K_2' = 0$; B: $K_3 = K_3'$, $K_2 = K_2'$; C: $K_3 = K_3'$, $K_2' = 0$, D: $K_3' = K_3$, $K_2' = K_3$. The top curve, E, is uncorrected. The concentration of cytochrome c varied from 0.9 to 1.2 x 10^{-5} M, concentration of $K_4Fe(CN)_6$ was 1.43 x 10^{-4} M. Temperature = 25°C, pH 7.0, and the ionic strength was 4.42 x 10^{-3} M.

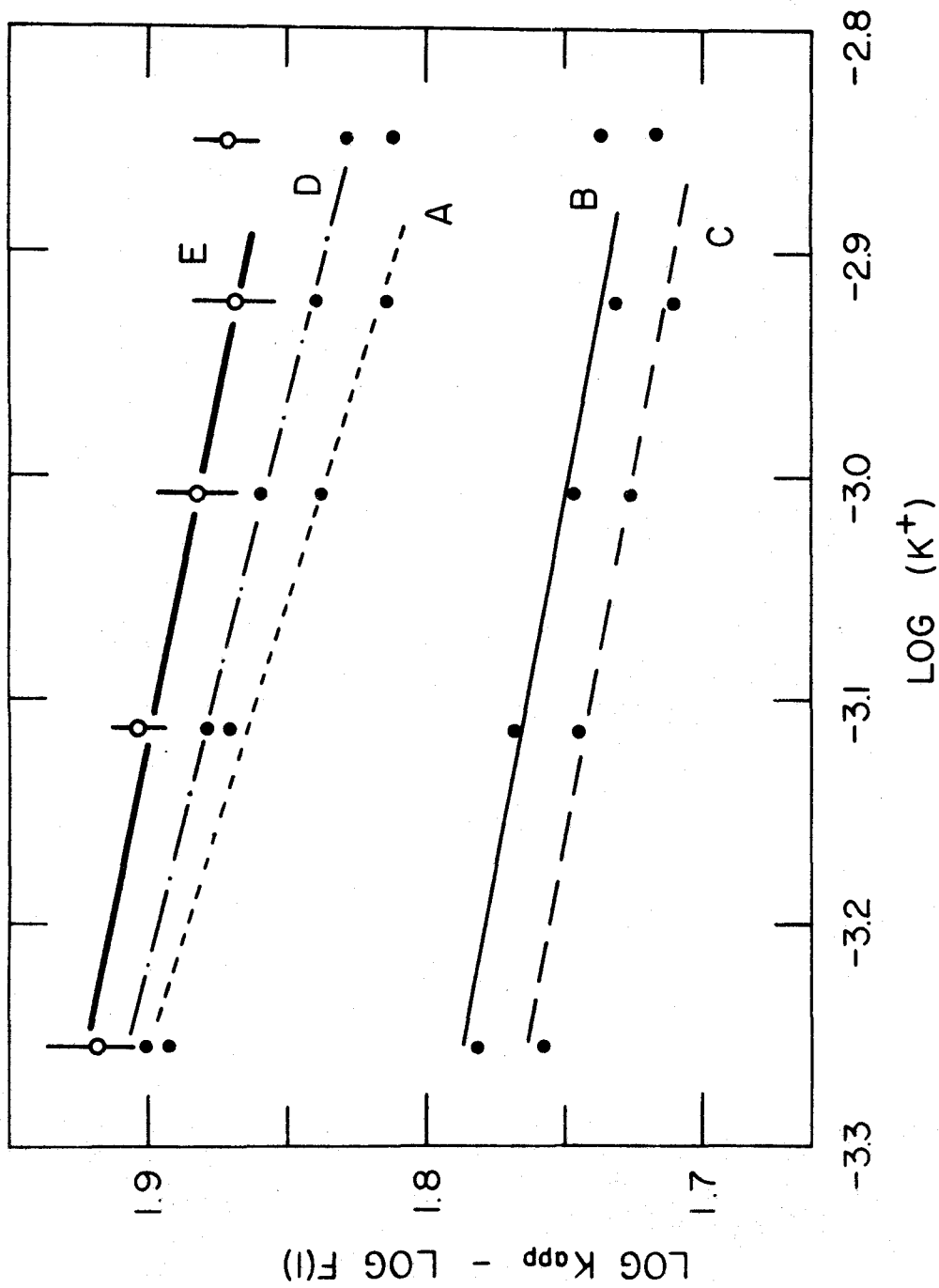

LOG K_{app} - LOG F(I)
1.9
1.8
1.7
LOG (K^+)
-2.8
-2.9
-3.0
-3.1
-3.2
-3.3
A
B
C
D
E

Table 4 gives values of the slope calculated from Figure 13.

TABLE 4

Effect of Correction for Cation Association on Potassium Binding to Cytochrome c

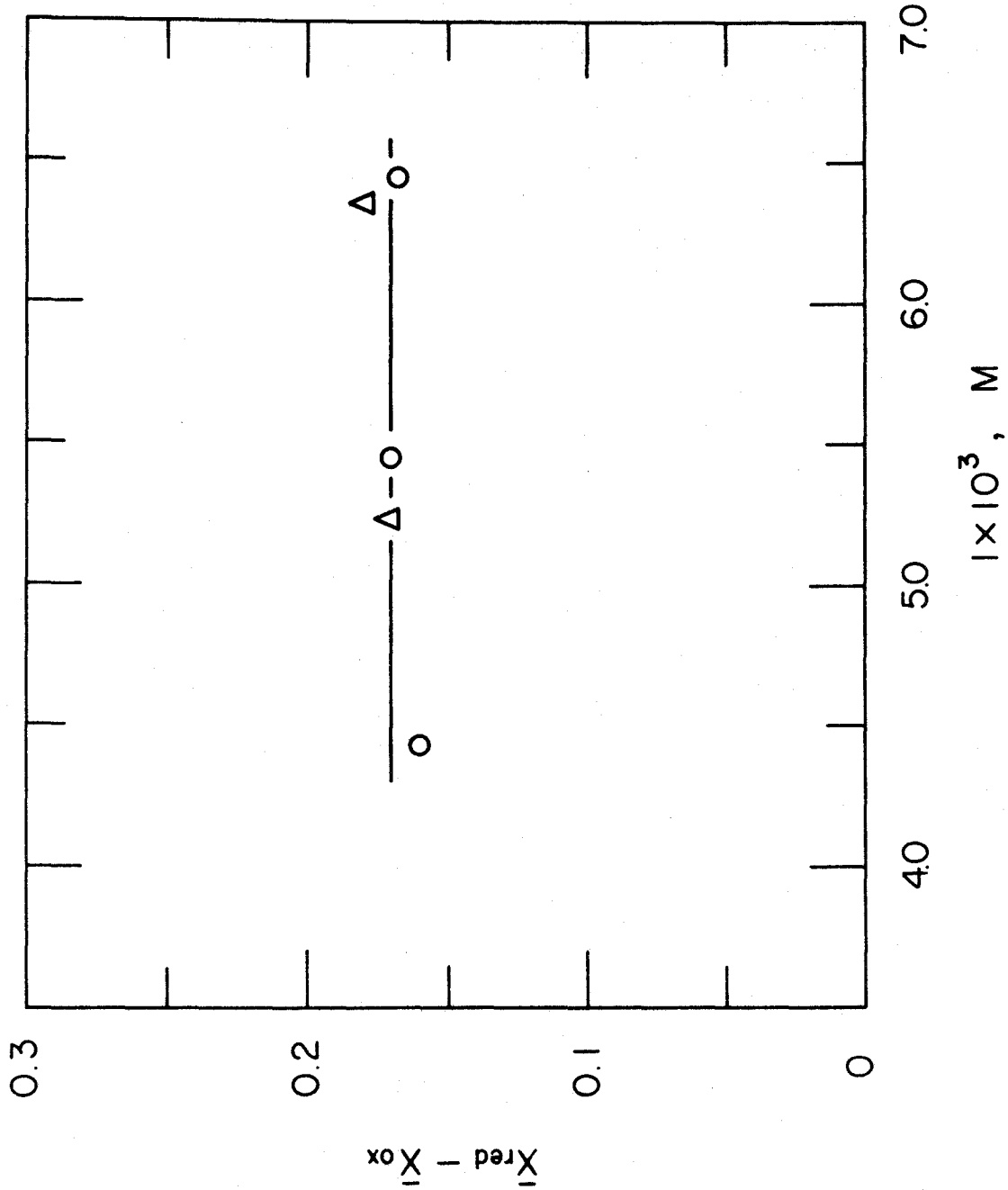
F(I) Correction	$(\bar{X}_{red} - \bar{X}_{ox})$
uncorrected	0.16
A	0.24
B	0.16
C	0.16
D	0.20

The important point is that the correction of the apparent equilibrium constant for cation binding to ferro- and ferricyanide increases slightly the differential binding to cytochrome c. Only if tris bound much more tightly to ferricyanide would $(\bar{X}_{red} - \bar{X}_{ox})$ be decreased. Available data cannot entirely rule out this possibility, but it would be less consistent with the observed ionic strength dependence of equilibrium constant (see page 49).

Figure 14 gives $(\bar{X}_{red} - \bar{X}_{ox})$ calculated from the slope of log K_{app} versus log (c^+) [without correction for F(I)] for different ionic strengths for both Na^+ and K^+. Within experimental uncertainties, we conclude that about 0.2 moles more of Na^+ or K^+ ions are bound on average to ferrocytochrome c

FIGURE 14

Effect of Ionic Strength on Potassium and Sodium Binding to Cytochrome c. Values of $(\overline{X}_{red} - \overline{X}_{ox})$ versus ionic strength for unmodified cytochrome c, obtained from the slope of uncorrected curves similar to Figure 13E, ○ for potassium binding, Δ for sodium binding. Experimental conditions were the same as in Figure 13.


0.3
0.2
0.1
0
$\bar{X}_{red} - \bar{X}_{ox}$
4.0
5.0
6.0
7.0
$I \times 10^3$, M

than to ferricytochrome c under these conditions (pH 7.0, T = 25°C, I about 0.005, c^+ about 10^{-3} M).

This value can be related to the number of potential binding sites available on ferrocytochrome c but not on ferricytochrome c, if additional restrictive assumptions are made. These are:

1) Ferricytochrome does not bind positive ions (Margoliash et al., 1970), therefore $K_{b_i} = 0$, i = 0 to n (equation C3).
2) Ferrocytochrome c binds either n cations or no cations and therefore: $K_{a_i} = 0$, i = 1 to n - 1.

By using these two additional assumptions we can derive an expression for n from equation C4.

$$K_{app} = KF(I) \frac{1}{1 + K_{an} (c^+)^n} \qquad (C9)$$

We define:

$$K_{app}^{nb} = KF(I)$$

This is the value of K_{app} when (c^+) is extrapolated to zero, i.e., in non-binding media.

Then:

$$K_{an}(c^+)^n = \frac{K_{app}^{nb} - K_{app}}{K_{app}} \qquad (C10)$$

If we plot log $[\frac{K_{app}^{nb} - K_{app}}{K_{app}}]$ vs log (c^+), the slope will give the value of n, the number of binding sites in ferrocytochrome c, and the intersection log K_{an}.

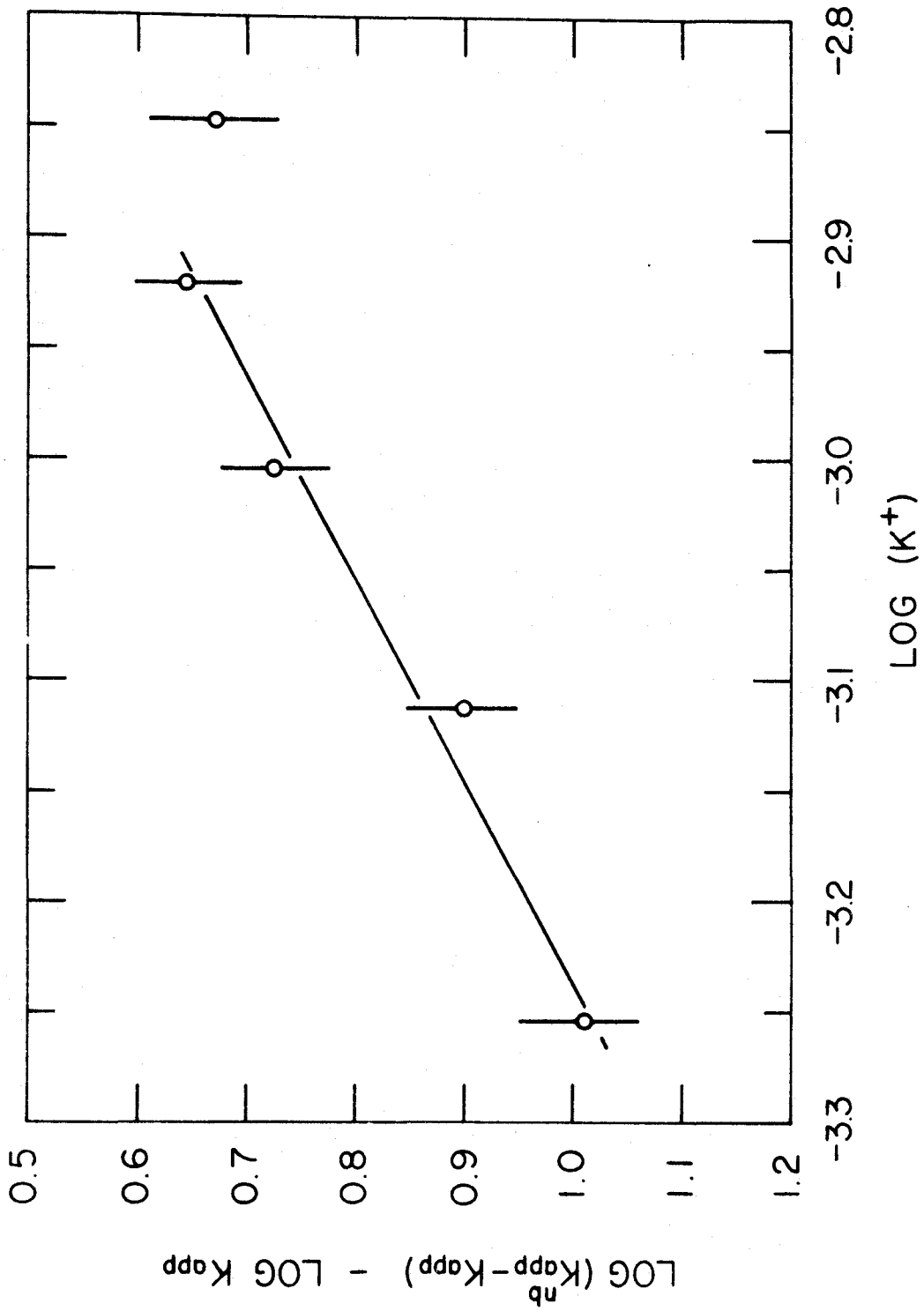
Differential binding of cations to ferri- and ferrocytochrome c is not a constant value, but probably decreases to zero at high concentrations of binding cation (see Figure 13). Therefore we make these additional assumptions only for measurements at intermediate cation concentrations, where the difference of binding between the two forms of cytochrome c is essentially constant.

Figure 15 gives an example of a plot according to equation C10 for unmodified cytochrome c at ionic strength, $I = 4.43 \times 10^{-3}$ M. The slope of the curve in Figure 15 and similar plots of the data for K^+ and Na^+ at other ionic strengths suggest that there is a single binding site for cations to cytochrome c and that the binding is much stronger to the reduced form of the protein.

FIGURE 15

Determination of the Number of Binding Sites and the Binding Constant for Cation Binding to Ferrocytochrome c.

The uncorrected data for the apparent equilibrium constant (see Figure 13E, experimental conditions are the same) plotted according to equation C10. n = the calculated slope is 1.1 and the binding constant $K_{an} = 3.6 \times 10^2\ M^{-1}$.


LOG (K^+)
-2.8
-2.9
-3.0
-3.1
-3.2
-3.3
LOG ($K_{app}^{nb} - K_{app}$) - LOG K_{app}
0.5
0.6
0.7
0.8
0.9
1.0
1.1
1.2

D. Binding of Cations (K^+) to Lysine-Modified Derivatives of Cytochrome c

The correction for cation binding to ferrocyanide and ferricyanide is independent of the nature of cytochrome c used. Therefore its uncertainty will not affect the comparison of the lysine-modified derivatives with unmodified cytochrome c. Figure 16 and Figure 17 give log K_{app} versus (K^+) for guanidinated cytochrome c, and 1-TNP-cytochrome c, respectively. The values plotted are uncorrected. It was impossible to obtain measurements at lower ionic strengths for modified proteins, probably because of their tendency to aggregate. The standard deviation from the mean was also larger.

Figure 18 shows the $(\bar{X}_{red} - \bar{X}_{ox})$ versus ionic strength for the modified proteins. The change in average number cations bound between ferro- and ferricytochrome c is the same as for the unmodified protein (Fig. 14).

To determine the number of binding sites, n, on the modified proteins, the data from Figures 16 and 17 were plotted according to the equation C10. Table 5 shows the values for n and K_{an} for cytochrome c and the modified derivatives.

The data from Figure 18 and Table 5 suggest that cation binding site on ferrocytochrome c is not affected by either modification of position 13 so that it no longer

FIGURE 16

The Binding of Potassium Ion to Guanidinated Cytochrome c.

Data were analyzed according to equation C4 [F(I) = 1] and can be compared with similar data for unmodified cytochrome c in Figure 13E. Experimental conditions: concentration of protein varied from 0.6 to 0.8 x 10^{-5} M, concentration of $K_4Fe(CN)_6$ was 1.65 x 10^{-4} M. Ionic strengths were: a) 5.9 x 10^{-3} M and b) 5.04 x 10^{-3} M. pH = 7.0, temperature = 25°C.

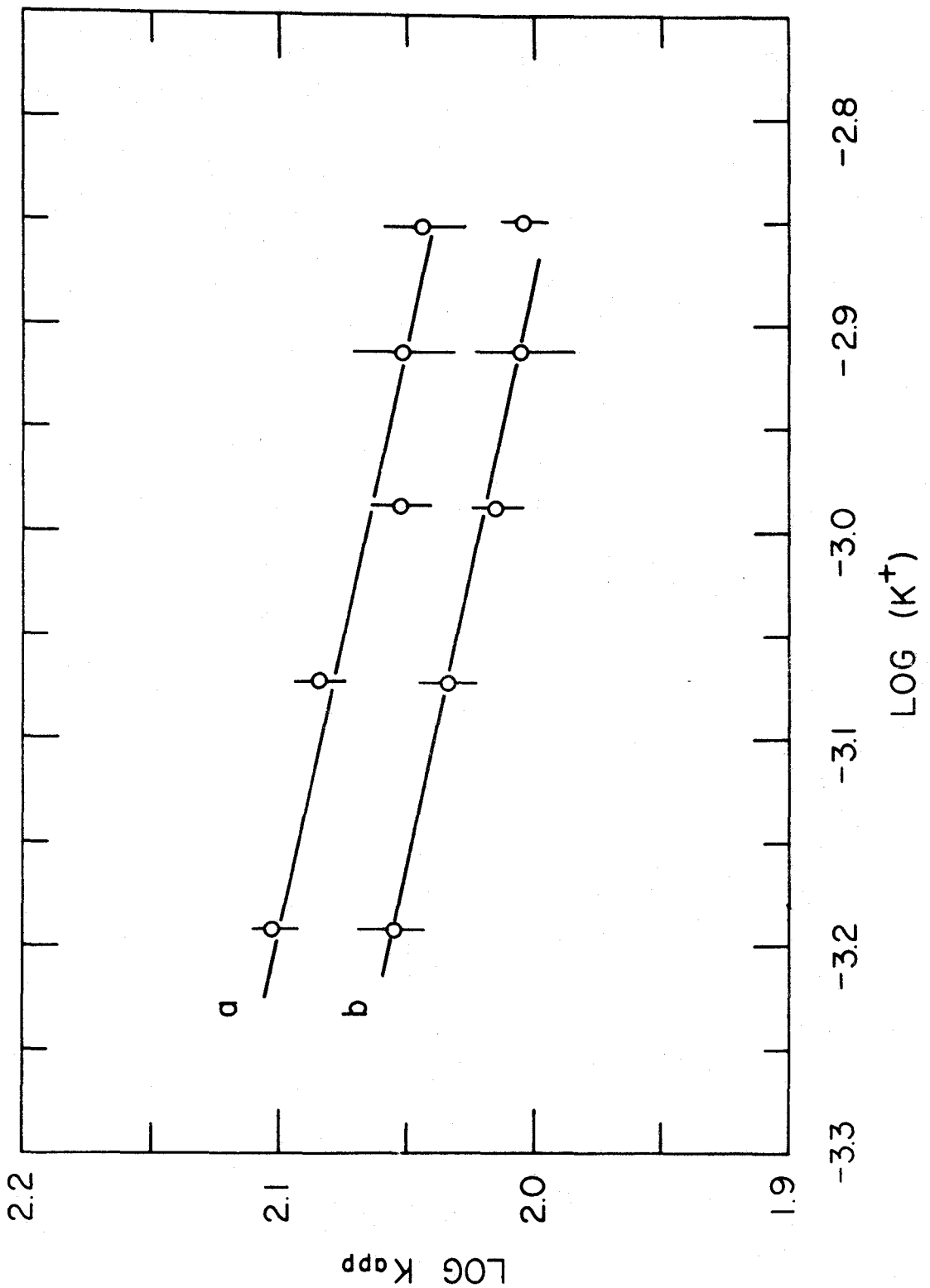

LOG K_{app}
2.2
2.1
2.0
1.9
-3.3
-3.2
-3.1
-3.0
-2.9
-2.8
LOG (K^+)
a
b

FIGURE 17

The Binding of Potassium Ion to 1-TNP-Cytochrome c.

Data were analyzed according to equation C4 [F(I) = 1] and can be compared with similar data in Figures 13E and 16. Concentration of protein 0.6 to 0.9×10^{-5} M. Concentration of $K_4Fe(CN)_6$ was 1.66×10^{-4} M. Ionic strengths were (a) 6.47×10^{-4} M and (b) 5.77×10^{-3} M. pH = 7.0; temperature 25°C.

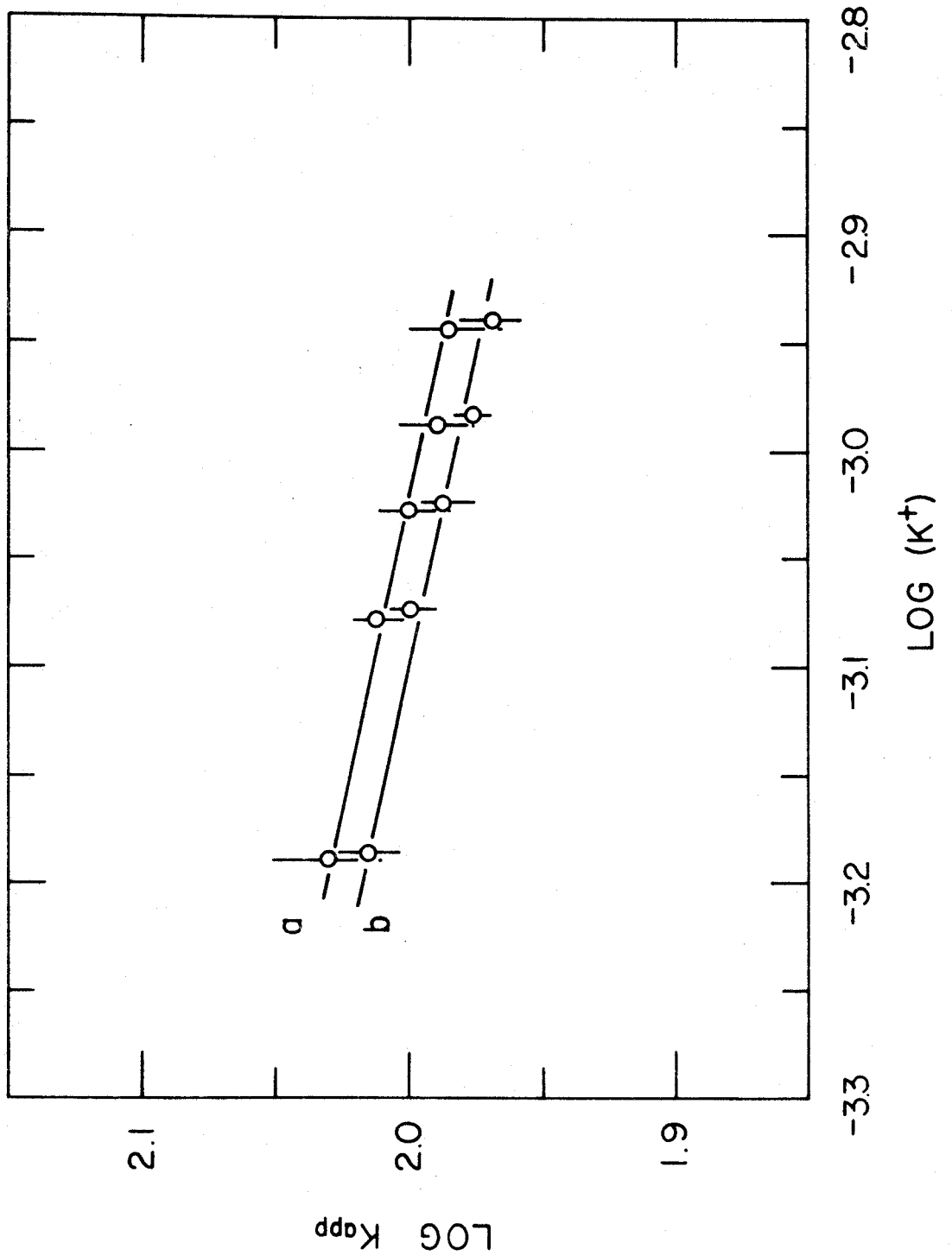
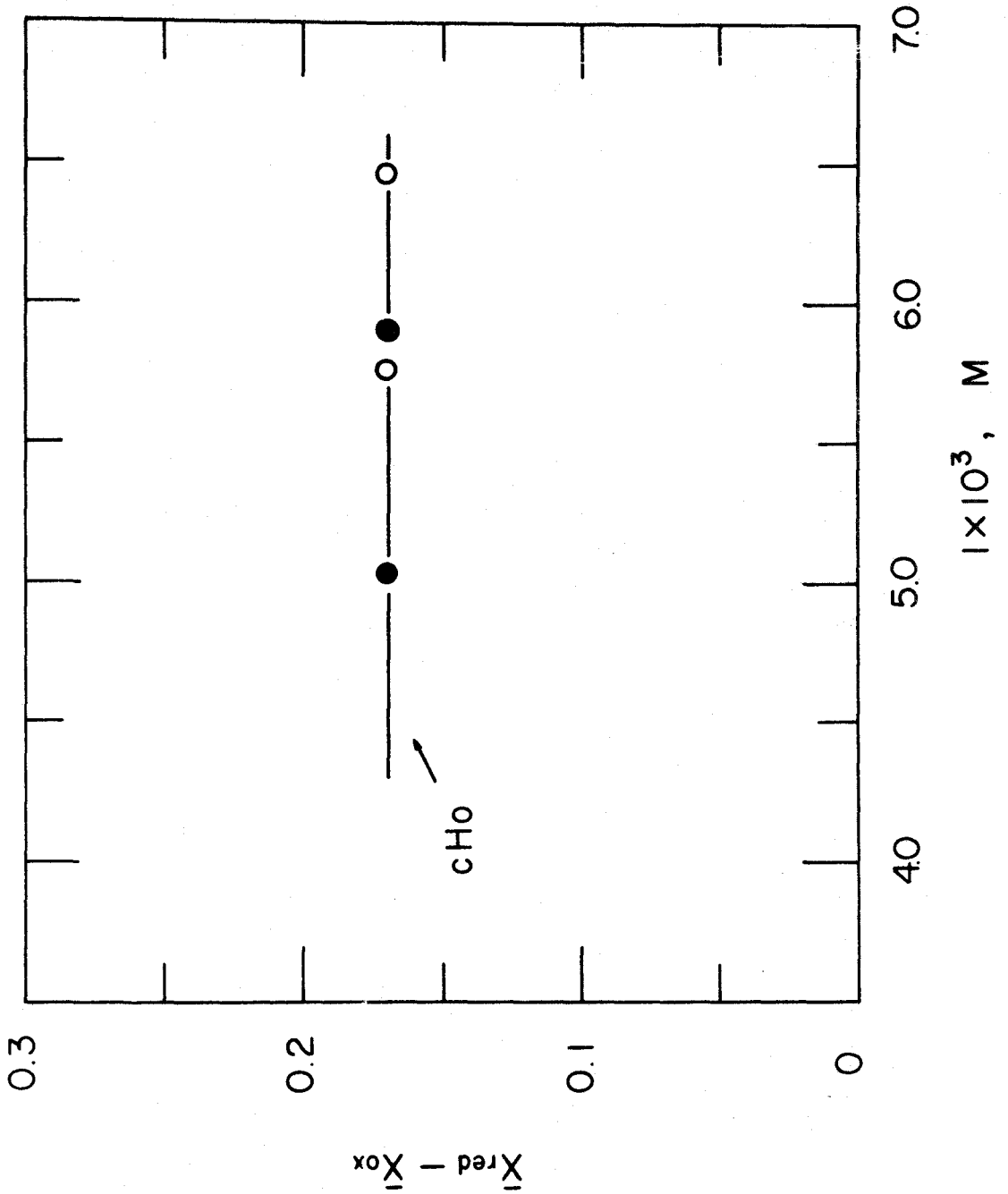

LOG K_{app}
2.1
2.0
1.9
LOG (K^+)
-2.8
-2.9
-3.0
-3.1
-3.2
-3.3
a
b

FIGURE 18

Effect of Ionic Strength on Potassium Binding to the Lysine Modified Derivatives of Cytochrome c.

The slopes from Figures 16 and 17 giving ($\overline{X}_{red} - \overline{X}_{ox}$) (the difference in average number of K^+ ions between reduced and oxidized forms of protein) versus ionic strength. Open circles, 1-TNP-cHo (Figure 17), solid circles, guanidinated-cHo (Figure 16). The line is for unmodified cytochrome c redrawn from Figure 14.


0.3
0.2
0.1
0
$\bar{X}_{red} - \bar{X}_{ox}$
4.0
5.0
6.0
7.0
$I \times 10^3$, M
cHo

contains a positive charge or conversion of all 19 lysine residues to homoarginine.

The strength of potassium binding is little affected by modification of lysine. Lysine residues seem to have little role in the cHo cation binding site.

TABLE 5

The Number of Binding Sites for Potassium Binding and the Binding Constants for Cytochrome c and the Lysine-Modified Derivatives

Protein	1×10^3 M	K_{an} [M^{-1}]	n
cHo^{II}	4.43	3.6×10^2	1.1
	5.0	9.1×10^2	1.2
	6.4	8.0×10^2	1.2
$Gn\text{-}cHo^{II}$	5.04	3.9×10^2	0.9
	5.9	1.4×10^2	1.1
$1\text{-}TNP\text{-}cHo^{II}$	5.77	2.0×10^2	1.0
	6.47	2.3×10^2	1.0

E. Binding of Negative Ions to Cytochrome c

In the section IIC, we derived the equation for the effect of cation binding to cytochrome c on the apparent equilibrium constant. The procedure for anion binding equation is analogous, and results in:

$$\frac{d[\log (\frac{K_{app}}{F(I)})]}{d \log (Cl^-)} = \bar{X}_{ox}' - \bar{X}_{red}' \qquad (E1)$$

where $\bar{X}_{ox}' - \bar{X}_{red}'$) represents the difference between average number of anions bound to oxidized and reduced form of the protein.

The experimental conditions for anion binding were such that the cation concentration of the solution was kept constant while cacodylate was replaced by the binding ion (Cl^-). The term F(I) thus remains constant for any given ionic strength, since we have assumed that anions do not form complexes with ferrocyanide or ferricyanide anions.

Figure 19 gives data for unmodified cytochrome c, plotted as required for E1. Results are uncorrected for F(I). From the slopes in Figure 19, we get ($\bar{X}_{ox}' - \bar{X}_{red}'$) for each ionic strength. These are given in Figure 20.

From Figure 20, we see that the difference in the average number of bound Cl^- ions between oxidized and reduced form of cytochrome c decreases with increasing ionic strength. In these experiments, ionic strength is

FIGURE 19

The Binding of Chloride to Cytochrome c.

The variation in the apparent equilibrium constant for unmodified cytochrome c with free Cl^- concentration. The data are plotted according to equation E1 with F(I) = 1. Experimental conditions: cytochrome c concentration varied from 0.9 to 1.2 x 10^{-5} M, concentration of $K_4Fe(CN)_6$ was 1.67 x 10^{-4} M. Ionic strengths were: (a) 6.19 x 10^{-3} M, (b) 5.31 x 10^{-3} M, (c) 3.94 x 10^{-3} M. pH = 7.0, temperature = 25°C.

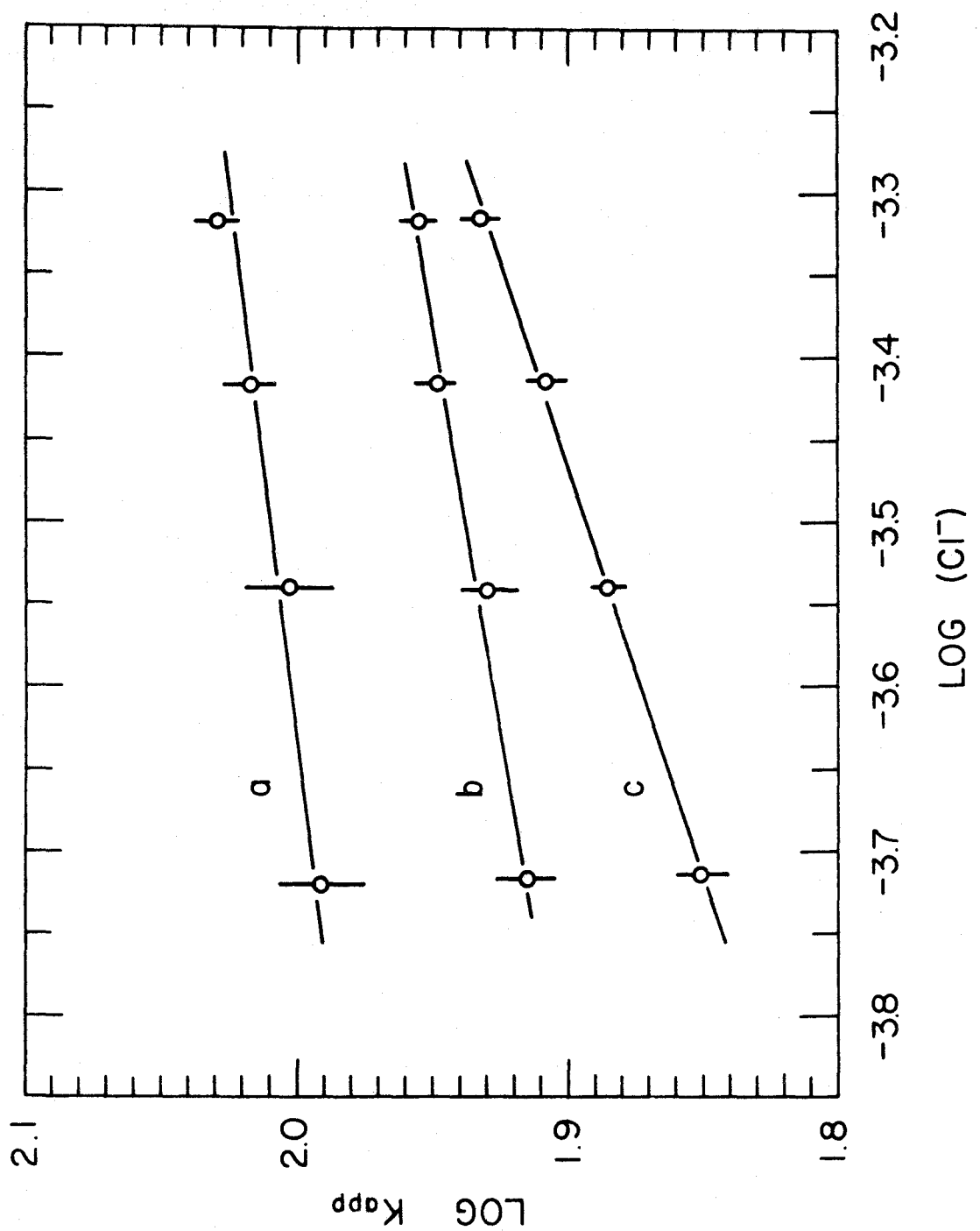
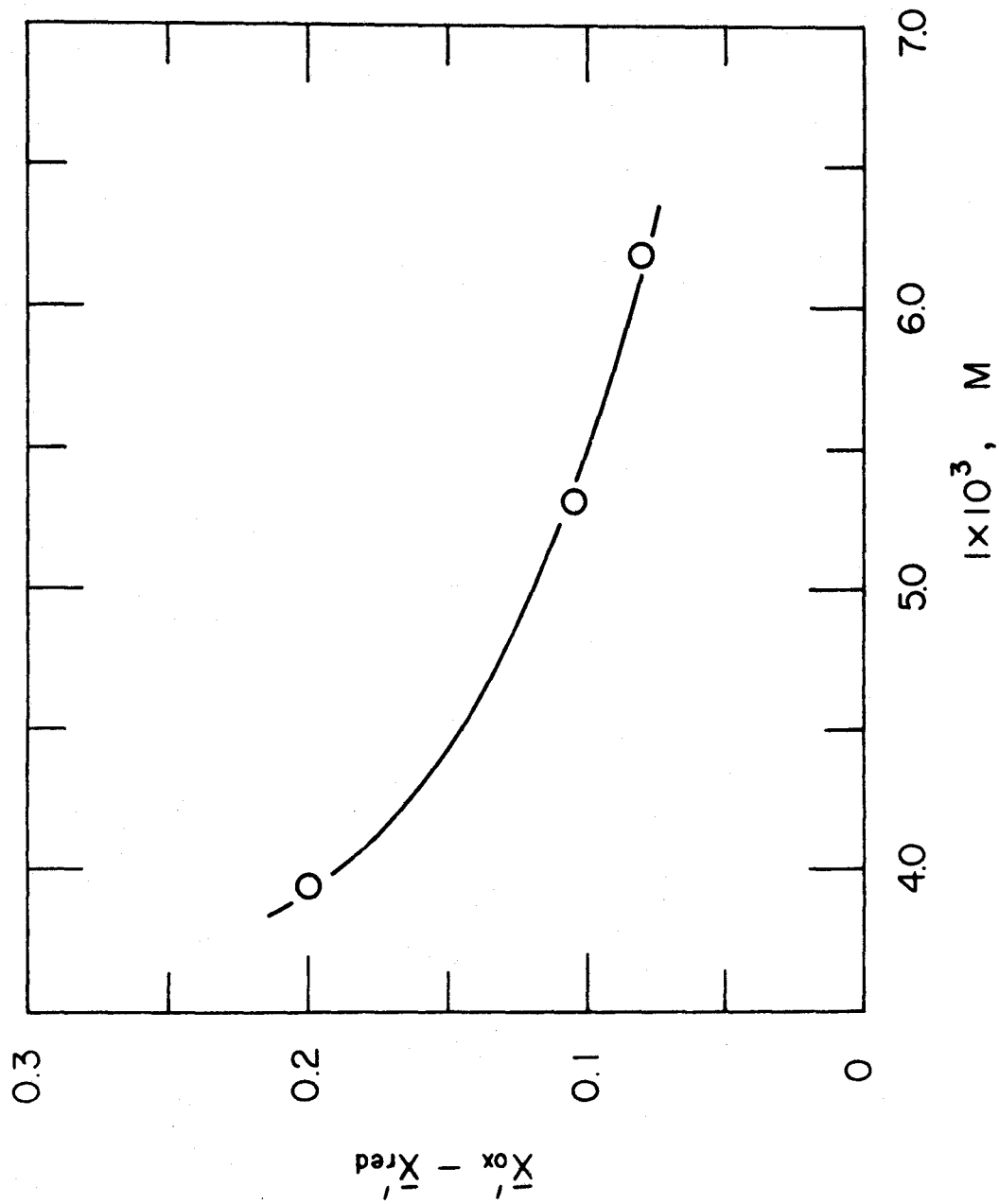

LOG k_{app}
2.1
2.0
1.9
1.8
-3.8
-3.7
-3.6
-3.5
-3.4
-3.3
-3.2
LOG (Cl^-)
a
b
c

FIGURE 20

Ionic Strength Dependence of Chloride Binding to Cytochrome c.

Values ($\bar{X}_{ox}' - \bar{X}_{red}'$) for unmodified cytochrome c obtained from the slopes of the curves in Figure 19 plotted against ionic strength. Experimental conditions as in Figure 19.


0.3
0.2
0.1
0
$\bar{\chi}'_{ox} - \bar{\chi}'_{red}$
4.0
5.0
6.0
7.0
$I \times 10^3$, M

increased by increasing the concentration of tris-cacodylate buffer. Since cacodylate anion is considered a non-binding ion, a competition between this ion and chloride for the same site should not be observed. Margalit and Schejter (1970) have observed the enthalpy changes for the reaction between ferricytochrome c and ferrocyanide, and they found that these decrease with ionic strength. The effect was explained by the fact that electrostatic repulsions at the surface of the molecule and the charge in the heme region are smaller at high ionic strengths due to the screening effect of counter ions. These electrostatic repulsions are important in stabilizing the configuration of protein in both oxidized and reduced states. At higher ionic strengths the differences in configuration between the oxidized and reduced protein could be smaller, which would explain the observed effect of ionic strength.

The value of $(\bar{X}_{ox}' - \bar{X}_{red}')$ can again be related to the potential binding sites available on ferricytochrome c, if we make additional assumptions, similar to those for potassium binding at low ion concentrations.

These assumptions are:

1. Ferrocytochrome c does not bind chloride ion (Margoliash *et al.*, 1970)

$$K_{a_i} = 0, \; i = 0 \text{ to } n'$$

2. Ferricytochrome c binds either n' Cl^- ions or none:

$$K_{b_i} = 0, \quad i = 1 \text{ to } n' - 1.$$

By using these two assumptions, and equation C4, we can derive the expression for n':

$$K_{app} = K\ F(I)\ \frac{1 + K_{bn'}\ (Cl)^{n'}}{1} \qquad (E2)$$

we defined:

$$K_{app}^{nb} = K\ F(I)$$

K_{app}^{nb} is the value of K_{app}, when concentration of Cl^- is extrapolated to zero.

$$K_{app} - K_{app}^{nb} = K_{app}^{nb}\ (Cl)^{n'}\ K_{bn} \qquad (E3)$$

If we plot log $(K_{app} - K_{app}^{nb})$ versus log (Cl^-), the slope will give n', the number of chloride binding sites in ferricytochrome c, and the intersection log $(K_{app}^{nb} \times K_{bn'})$, from which we can evaluate $K_{bn'}$, the binding constant of chloride ion to ferricytochrome c.

Figure 21 gives an example of such a plot according to the equation E3 for unmodified cytochrome c. From similar plots for other ionic strengths n' and binding constants of chloride to cHo^{III} were evaluated (see Table 6).

FIGURE 21

Determination of the Number of Binding Sites and the Binding Constant for Chloride Binding to Ferricytochrome c. The uncorrected data for the apparent equilibrium constant (see Figure 19c, experimental conditions are the same) plotted according to equation E3. n' = the calculated slope is 1.8, $K_{an'} = 2.8 \times 10^5\ M^{-1}$.

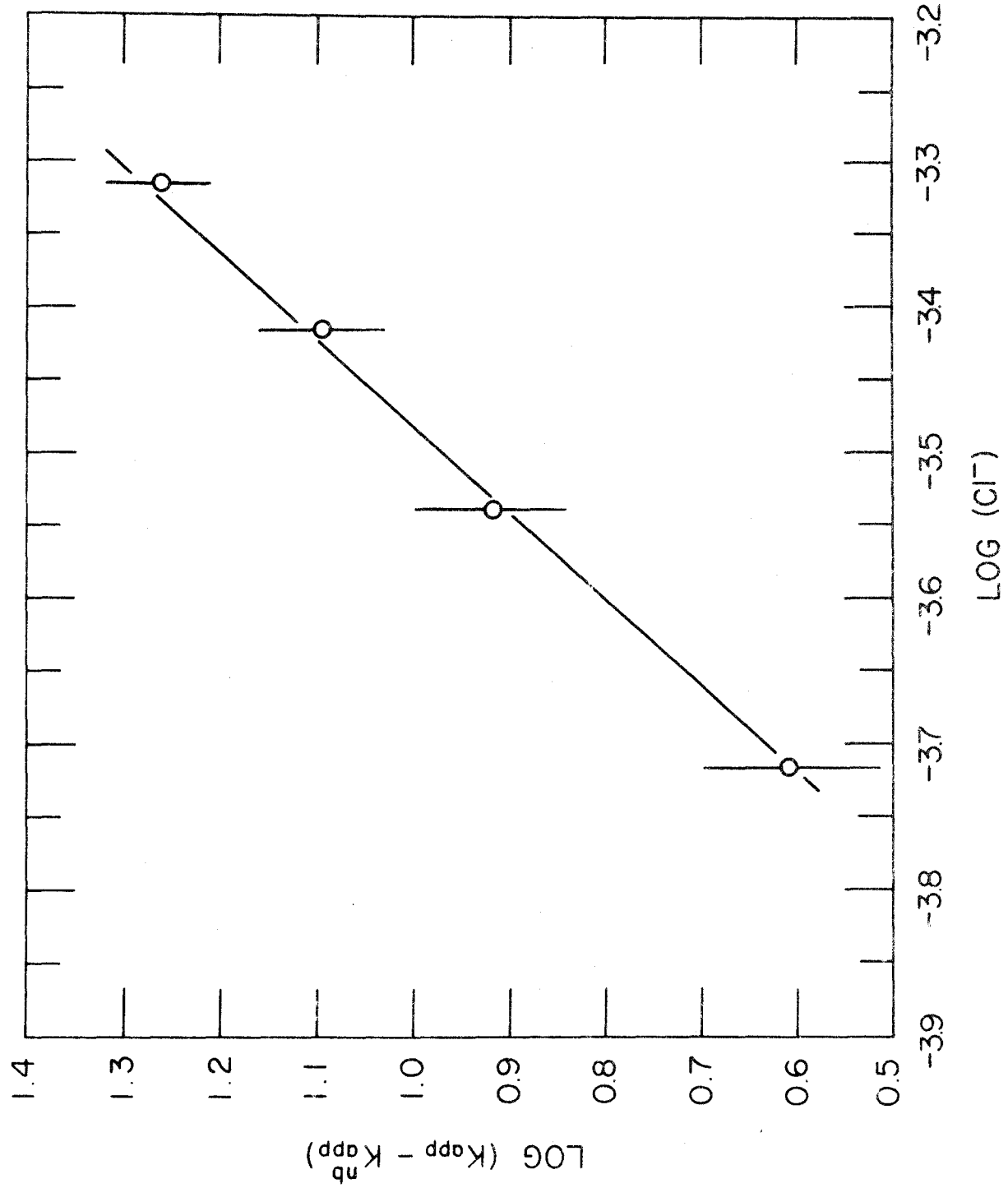

LOG ($K_{app} - K_{app}^{nb}$)
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
LOG (Cl^-)
-3.2
-3.3
-3.4
-3.5
-3.6
-3.7
-3.8
-3.9

TABLE 6

The Number of Binding Sites for Chloride Binding and the Binding Constants for Cytochrome c at Different Ionic Strengths

$I \times 10^3$ [M]	$K_{bn'}$ [M^{-1}]	n'
3.94	2.8×10^5	1.8
5.31	3.5×10^2	0.95
6.19	1.2×10^2	0.85

From the above results we concluded that there are two different binding sites for chloride present in the cytochrome c molecule, and the binding is much stronger to the oxidized form of the protein. These results are consistent with the observations of Schejter and Margalit (1970) who have measured the binding of chloride at ionic strength 0.004 M and estimated the values n' = 1.6 and $K_{bn'} = 5 \times 10^5$ [M^{-1}]

F. Binding of Cl^- to Modified Derivatives of Cytochrome c

Modifications of cytochrome c was done in two different ways. First so that it retained the positive charge (Gn-cHo); second, specifically at residue 13, removing its positive charge (1-TNP-cHo). We examined the effect on chloride binding. Experiments planned with TFA-cHo in which all lysines had lost their positive charge were unsuccessful due to the low reduction potential of the product.

Figures 22 and 23 give log K_{app} (uncorrected) versus log (Cl^-) for both modified derivatives.

Figure 24 shows ($\bar{X}_{ox}' - \bar{X}_{red}'$) versus ionic strength for the modified proteins. ($\bar{X}_{ox}' - \bar{X}_{red}'$) represents the difference in average number of Cl^- ions bound to oxidized and reduced form of cytochrome c. Results in Figure 24 are compared to the results obtained for unmodified protein (solid line in Figure 24).

In the ionic strength range examined, there remains a small ionic strength effect but much less than that for unmodified cytochrome c. In the case of 1-TNP-cHo at an ionic strength of .004 M, the value ($\bar{X}_{ox}' - \bar{X}_{red}'$)is decreased about 50%. Modification of all 19 lysines decreases this only slightly further. Both modified derivatives still differentially bind chloride ion between oxidized and reduced forms but less than the native protein. In order to determine

FIGURE 22

The Binding of Chloride to Guanidinated Cytochrome c.

Data were analyzed according to equation E1 (F(I) = 1). These can be compared with similar data for unmodified cytochrome c in Figure 19. Experimental conditions: Gn-cHo concentration in the range 0.6 to 0.9 x 10^{-5} M, $K_4Fe(CN)_6$ was 1.65 x 10^{-4} M. Ionic strengths: (a) 6.2 x 10^{-3} M, (b) 5.27 x 10^{-3} M, (c) 4.38 x 10^{-3} M. pH = 7.0, temperature = 25°C.

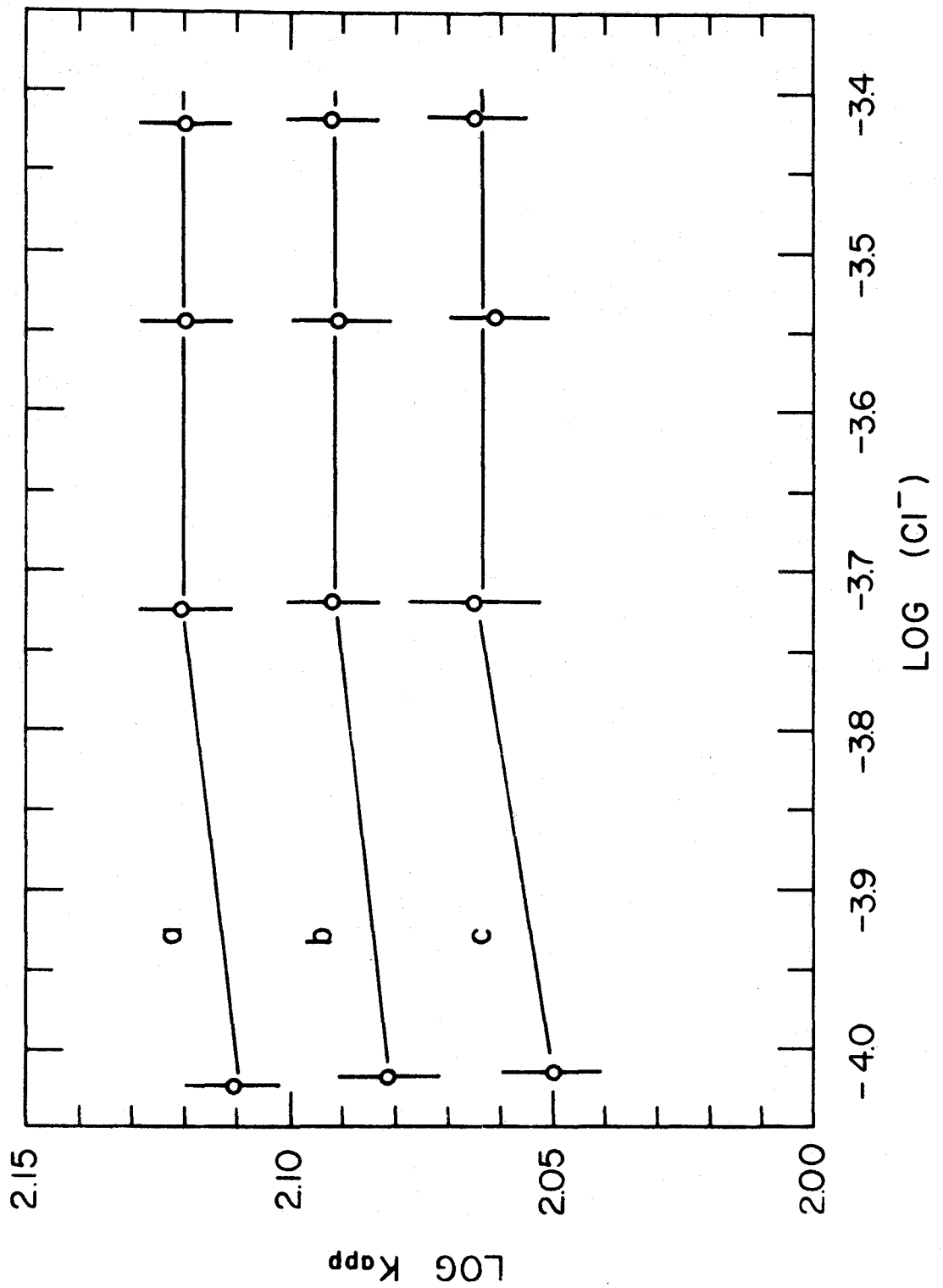

2.15
2.10
2.05
2.00
LOG k_{app}
-4.0
-3.9
-3.8
-3.7
-3.6
-3.5
-3.4
LOG (Cl^-)
a
b
c

FIGURE 23

The Binding of Chloride to 1-TNP-Cytochrome c.

Data were analyzed according to equation E1 [F(I) = 1] and may be compared with similar data with unmodified cytochrome c (Figure 19), and guanidinated cytochrome c (Figure 22). 1-TNP-cHo concentration from 0.6 to 0.9 x 10^{-5} M, $K_4Fe(CN)_6$ was 1.64 x 10^{-4} M. Ionic strengths were: (a) 5.95 x 10^{-3} M, (b) 5.13 x 10^{-3} M, (c) 3.86 x 10^{-3} M. pH = 7.0, temperature = 25°C.

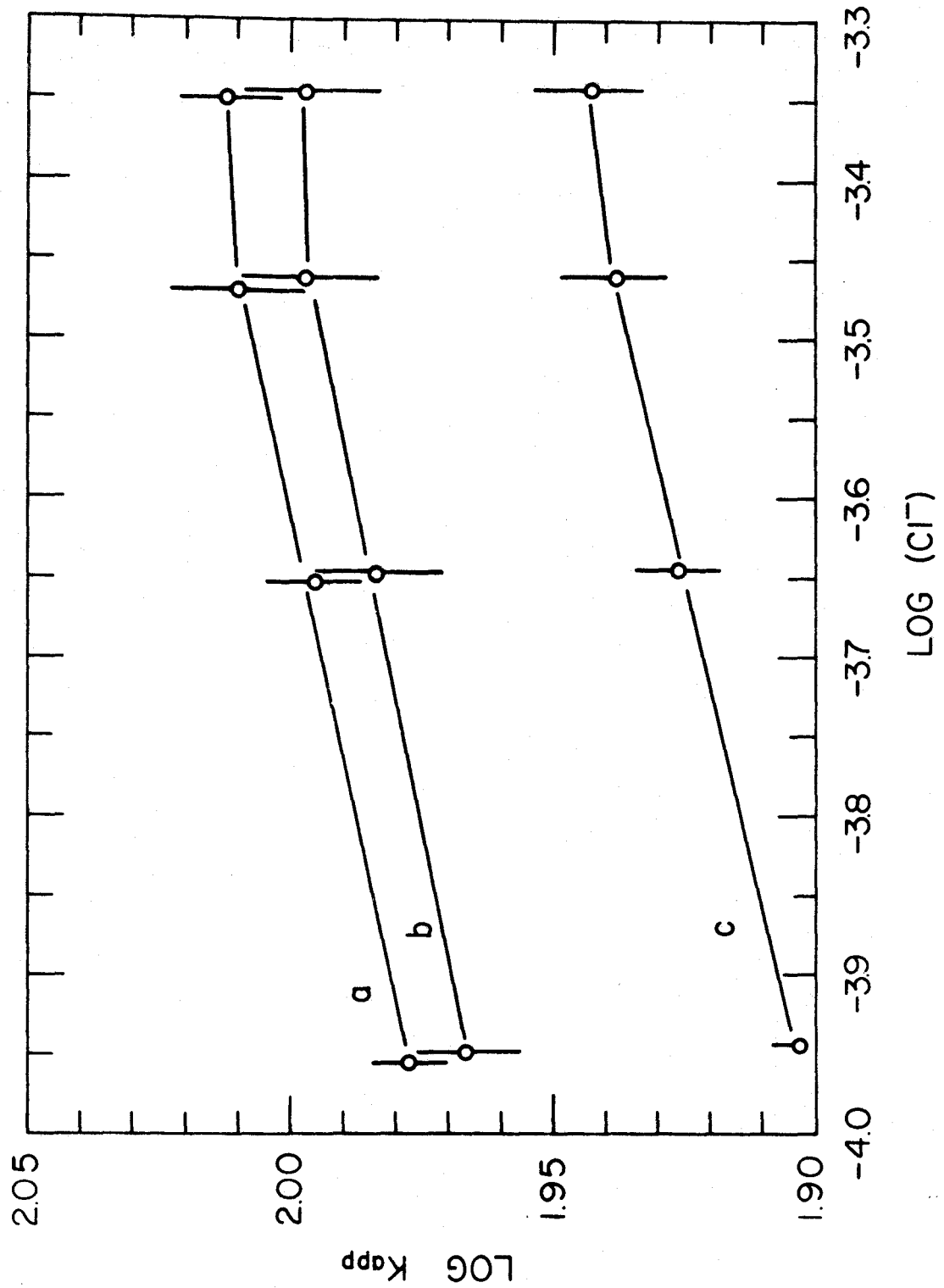
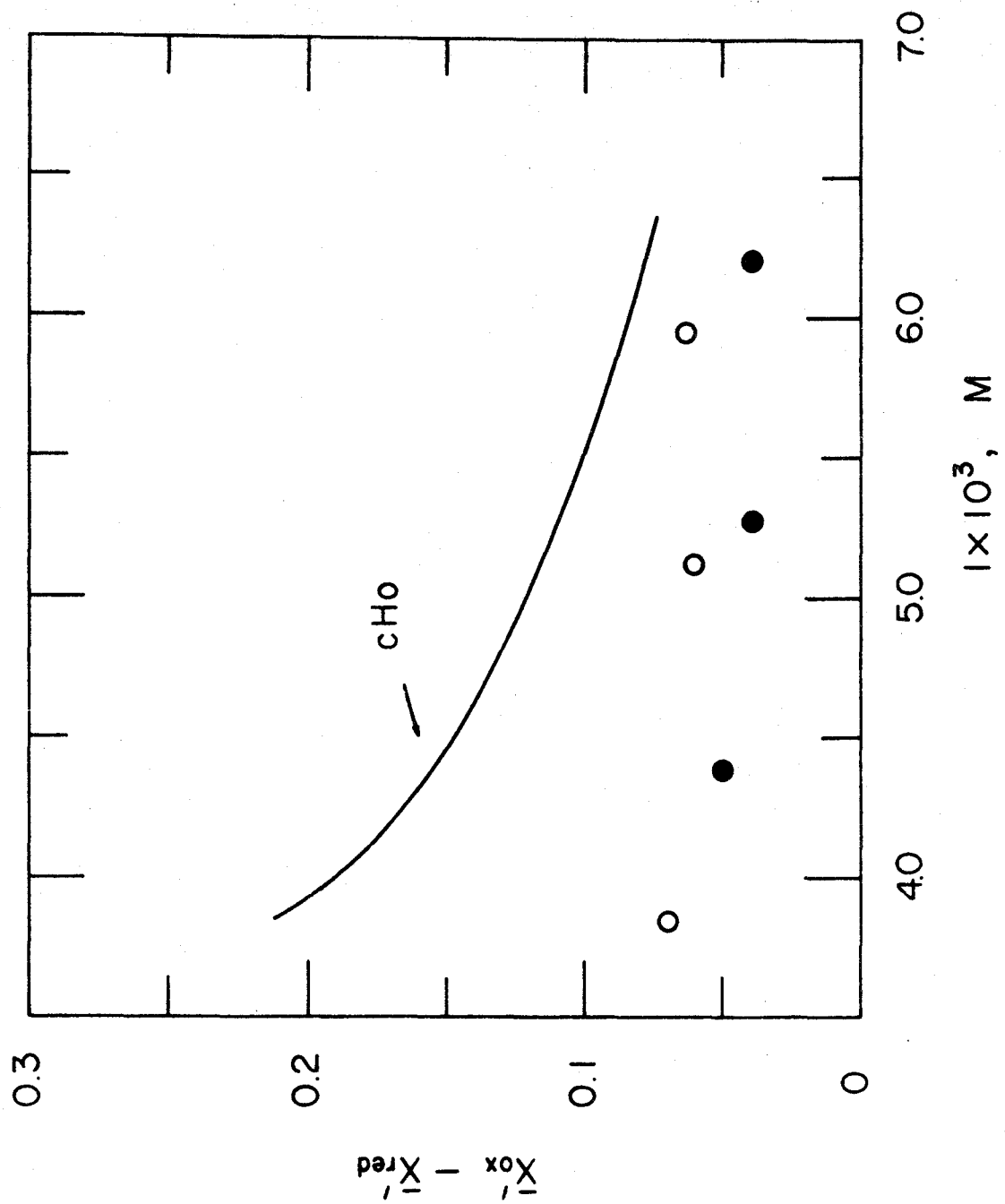

2.05
2.00
1.95
1.90
LOG k_{app}
-4.0
-3.9
-3.8
-3.7
-3.6
-3.5
-3.4
-3.3
LOG (Cl^-)
a
b
c

FIGURE 24

The Effect of Ionic Strength on Chloride Binding to Lysine-Modified Derivatives of Cytochrome c. The values of $\bar{X}_{ox}' - \bar{X}_{red}'$ for 1-TNP-cHo (O) and guanidinated-cHo (●), obtained from the initial slopes of the curves of Figures 22 and 23, plotted against the ionic strength. The line is for unmodified cytochrome c redrawn from Figure 20.


0.3
0.2
0.1
0
$\bar{X}'_{ox} - \bar{X}'_{red}$
cHo
4.0
5.0
6.0
7.0
1×10^3, M

the number of binding sites for Cl^-, the data from Figure 22 and 23 were analyzed according to equation E3 and values of n' and $K_{bn'}$ evaluated. Table 7 gives n' and $K_{bn'}$ for modified proteins (Gn-cHo^{III} and 1-TNP-cHo^{III}) estimated for different ionic strengths.

TABLE 7

Binding Constants and Number of Chloride Binding Sites for Lysine Modified Derivatives

cytochrome c	I x 10^3 [M]	K_{bn^1} [M^{-1}]	n'
Gn cHo	4.38	7.2 x 10^1	0.8
	5.27	3.3 x 10^1	0.7
	6.2	8.7 x 10^1	0.9
1 TNP cHo	3.85	6.1 x 10^1	0.7
	5.13	3.7 x 10^1	0.7
	5.95	1.05 x 10^2	0.8^5

Data from Figure 24 and Table 7 suggest that chloride ion binding is changed considerably upon modification of all lysines and nearly as much by trinitrophenylation of a single residue (number 13). The data are consistant with retention of a single Cl^- binding site in the lysine-modified derivatives which is independent of ionic strength within the experimental error. These results suggest that one of the binding sites for Cl^- ion involves a single lysine

residue, probably number 13, and that, even though electrostatic interaction probably plays a role, the exact structure of the residue is necessary because of the results for guanidinated cytochrome in which lysine 13 is changed to a positively charged homoarginine.

IV. SUMMARY AND CONCLUSIONS

1. Guanidination

The procedure for guanidination of cytochrome c modifies all 19 lysine residues (Table 1). The configuration of the surface remains essentially the same as that of the unmodified protein, as shown by the perturbation spectra in 20% ethylene glycol (Fig. 1). The guanidinated derivative is more subjected to aggregation than native cytochrome c (Morton, unpublished) and this is presumably the cause of the decrease in reduction potential observed at either the lower ionic strength or upon storage of the purified protein. The reduction potential of pure unaggregated guanidinated cytochrome c seems to be slightly lower than that of cytochrome c (Fig. 12). This effect could, however, be due to a slight differential binding of cacodylate anions by ferricytochrome which is lost when the protein is guanidinated.

2. Trifluoroacetylation

Modification of ε-NH_2 groups in cytochrome c with S-ethyltrifluoroacetate resulted in a derivative with about 90% of lysine groups modified (Table 1). The modified protein shows some differences in the configuration of the surface (Fig. 1). The reduction potential of TFA-cHo is much lower

than cytochrome c (Table 3) presumably as a result of electrostatic distortion of the native configuration. This prevented measurements for ion binding of this derivative.

3. Trinitrophenylation

Trinitrobenzene sulfonate reacted under specific conditions with one most reactive amino group in cytochrome c molecule. A derivative with an average of one lysine residue modified has been isolated, and this residue is presumably lysine 13 (Morton, unpublished). The difference in absorption spectra between 1-TNP-cHo^{III} and cHo^{III} correspond to the addition of a single TNP group (Fig. 3). The difference spectrum between reduced forms was not obtained because the reduction of TNP group was more favourable than the reduction of heme. Alkaline pH titration of 1-TNP-cHo^{III} showed a similar transition from type III to type IV to that for the unmodified protein, characterized by the loss of 695 nm absorption band (Figure 5). The difference between 1-TNP-cHo^{III} and cHo^{III} pH-induced spectra changes (Figs. 5,6,7 and 8) is similar to the change observed upon dithionite reduction of the TNP group (Fig. 4) suggesting that this group is reduced at alkaline pH by the unknown reducing agent generated in ferricytochrome c solutions at high pH (Brady and Flatmark, 1971).

The reduction potential of 1-TNP-cHo is, like Gn-cHo,

slightly lower than that of cytochrome c (Fig. 12).

4. The Effect of Ionic Strength on Reaction Between Ferrocyanide and Ferricytochrome c

Measurements of K_{app} in assumed non-binding media (tris-cacodylate buffer) indicate that cytochrome c behaves at low ionic strength in a typical Debye-Hückel fashion (Figure 12). If we assume the net change of charge upon the reduction from 10.5 to 9.5, we obtain the standard reduction potential of the protein $E_{o_2}' = 0.284$ V. Results cannot be unambiguously interpreted because of uncertainties in binding constants for tris cation with ferrocyanide and more importantly with ferricyanide. The results suggest, however, that tris does not strongly discriminate between these two agents.

5. Cation Binding

The interpretation of cation binding experiments is not completely defined because of the unknown magnitude of the association constant between tris and ferricyanide. The results (Table 5) and the electrophoretically measured binding of K^+ and Na^+ to ferrocytochrome c (Margoliash et al., 1970) suggest that the uncorrected data give reasonable estimation of cation binding (that is ignoring cation binding to ferro-, ferricyanide system). In this case, we suggest a single cation binding site with a stronger association

constant in ferro- than ferricytochrome c. Neither guanidination of all lysines nor trinitrophenylation of one (lysine 13) affects this cation binding site (Table 5, Figure 18).

6. Anion Binding

The results for unmodified cytochrome c indicate that there are 2 binding sites for chloride with a stronger association constant in ferricytochrome c than in ferrocytochrome c (Fig. 20). The binding of chloride is ionic strength dependent (Fig. 19), possibly due to electrostatic repulsions between charges at the surface of the molecule and the heme group.

Chloride binding is dramatically affected by modification of lysine residues. The changes are essentially the same whether all 19 lysine residues are converted to homoarginine, or if only the residue number 13 is trinitrophenylated (Fig. 24). These results suggest that one of the binding sites for chloride ions involves a single lysine residue, probably residue number 13.

It has been suggested (Margoliash et al., 1970) that cytochrome c is a carrier for some of the ions, which require specific translocation mechanisms in and out of the mitochondrial matrix. Some of these are typical, non-penetrating anions, and cations which can be accumulated in

mitochondria (Klingenberg, 1970), while others undergo an exchange diffusion process through the inner mitochondrial membrane. If this hypothesis is correct, the cytochromes c may represent a control point between the activity of mitochondria and the metabolism of the rest of the cell.

It would be useful to obtain quantitative data on the binding of divalent ions to cytochrome c, since those ions are also known to accumulate in mitochondria. The binding of ATP, ADP, and phosphate, which have a central influence on metabolic rates, may be a physiological mechanism for controlling the rates of oxidation and reduction of cytochrome c. Data for those ions are not yet available. Further it would be interesting to compare binding properties of cytochrome c from different species. The lysine residue number 13 in horse heart cytochrome c is not invariable, therefore it would be interesting to examine the chloride binding properties of cytochrome c from the species which do not contain this residue, to show the possible differences. If differences in ion binding were found, one would eventually be able to explain how the structural variations have been adapted to the different metabolic activities of a particular species.

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VI. APPENDIX

Table of the Apparent Equilibrium Constants Used in Section 2

I. Values of K_{app} in Non-Binding Media

(a) Cytochrome c:

K_{app}	I x 10^3 [M]	$[Fe(CN)_6^{4-}] \times 10^4$ [M]	$(T^+) \times 10^3$ [M]
68.6 ± 1.5	3.94	1.66	2.27
75.3 ± 2.8	4.3	1.59	2.72
80.4 ± 0.8	5.2	1.56	3.64
89.2 ± 1.3	6.3	1.56	4.8
100.0 ± 1.2	7.1	1.66	5.45

(b) Guanidinated Cytochrome c:

K_{app}	I x 10^3 [M]	$[Fe(CN)_6^{4-}] \times 10^4$ [M]	$(T^+) \times 10^3$ [M]
101.6 ± 1.6	3.49	1.70	1.82
110.9 ± 2.2	4.32	1.70	2.73
119.5 ± 1.4	6.06	1.70	4.55
129.6 ± 1	6.88	1.70	5.45

(c) 1 TNP Cytochrome c:

K_{app}	I x 10^3 [M]	$[Fe(CN)_6^{4-}] \times 10^4$ [M]	$(T^+) \times 10^3$ [M]
80.4 ± 1.1	3.42	1.64	1.82
93.4 ± 2.2	4.3	1.64	2.73
100.5 ± 1.3	5.14	1.64	3.64
106.8 ± 1.9	5.97	1.64	4.55
116.5 ± 1.2	6.79	1.64	5.45

(d) TFA Cytochrome c:

$K_{app} \times 10^{-4}$	I x 10^3 [M]	$[Fe(CN)_6^{4-}] \times 10^4$ [M]	$(T^+) \times 10^3$ [M]
2.46 ± 0.2	9.32	4.86	4.46
1.95 ± 0.2	10.14	4.86	5.28
1.75 ± 0.1	11.66	4.86	6.92
1.57 ± 0.1	13.32	4.86	8.47

II. K_{app} in K^+ Binding Media

(a) Cytochrome c:

$I = 4.43 \times 10^{-3}$ M

K_{app}	$(K^+) \times 10^4$ [M]	$[Fe(CN)_6^{4-}] \times 10^4$ [M]	$(T^+) \times 10^3$ [M]
82.4 ± 1.5	5.7	1.42	3.01
80.4 ± 1.2	7.87	1.42	2.79
76.1 ± 1.6	10.04	1.42	2.58
73.7 ± 1.2	12.22	1.42	2.36
74.7 ± 1.2	14.39	1.42	2.14

$I = 5.44 \times 10^{-3}$ M

K_{app}	$(K^+) \times 10^4$ [M]	$[Fe(CN)_6^{4-}] \times 10^4$ [M]	$(T^+) \times 10^3$ [M]
87.4 ± 3.1	5.7	1.42	4.02
82.4 ± 2.1	7.87	1.42	3.79
76.7 ± 1.5	10.04	1.42	3.59
73.5 ± 1.1	12.22	1.42	3.37
75.0 ± 1.2	14.37	1.42	3.15

$I = 6.45 \times 10^{-3}$ M

K_{app}	$(K^+) \times 10^4$ [M]	$[Fe(CN)_6^{4-}] \times 10^4$ [M]	$(T^+) \times 10^3$ [M]
91.5 ± 2.1	5.7	1.42	5.02
85.6 ± 1.2	7.87	1.42	4.81
83.6 ± 2.2	10.04	1.42	4.59
79.6 ± 1	12.22	1.42	4.37
78.3 ± 1	14.37	1.42	9.15

(b) Guanidinated Cytochrome c:

$I = 5.04 \times 10^{-3}$ M

K_{app}	$(K^+) \times 10^4$ [M]	$[Fe(CN)_6^{4-}] \times 10^4$ [M]	$(T^+) \times 10^3$ [M]
114.4 ± 2.7	6.56	1.69	3.4
108.7 ± 2.4	8.55	1.69	3.2
104.2 ± 2.0	10.54	1.69	3.0
101.7 ± 3.6	12.54	1.69	2.8
101.4 ± 2.0	14.55	1.69	2.6

(b) Guanidinated Cytochrome c (cont'd):

$I = 5.9 \times 10^{-3}$ M

K_{app}	$(K^+) \times 10^4$ [M]	$[Fe(CN)_6^{4-}] \times 10^4$ [M]	$(T^+) \times 10^3$ [M]
127.1 ± 2.2	6.56	1.69	4.25
120.5 ± 2.5	8.54	1.69	4.05
113.2 ± 1.8	10.54	1.69	3.86
113.2 ± 3.5	12.48	1.69	3.66
111.5 ± 2	14.55	1.69	3.46

(c) Trinitrophenylated Cytochrome c:

$I = 5.77 \times 10^{-3}$ M

K_{app}	$(K^+) \times 10^4$ [M]	$[Fe(CN)_6^{4-}] \times 10^4$ [M]	$(T^+) \times 10^3$ [M]
103.8 ± 3.3	6.64	1.66	4.11
100.2 ± 2.2	8.62	1.66	3.91
97.3 ± 1.8	9.61	1.66	3.81
94.5 ± 1.6	10.60	1.66	3.71
93.1 ± 3.4	11.58	1.66	3.62

$I = 6.47 \times 10^{-3}$ M

K_{app}	$(K^+) \times 10^4$ [M]	$[Fe(CN)_6^{4-}] \times 10^4$ [M]	$(T^+) \times 10^3$ [M]
107.9 ± 5.4	6.64	1.66	5.61
102.8 ± 3.2	8.61	1.66	4.91
99.9 ± 3.6	9.60	1.66	4.86
97.4 ± 4.1	10.57	1.66	4.76
96.4 ± 3.9	11.55	1.66	4.67

(d) cHo in Na^+ Binding Media

$I = 5.2 \times 10^{-3}$ M

K_{app}	$(Na^+) \times 10^4$ [M]	$[Fe(CN)_6^{4-}] \times 10^4$ [M]	$(T^+) \times 10^3$ [M]
81.3 ± 0.9	0	1.56	3.64
79.5 ± 1	1.26	1.56	3.51
77.4 ± 1.5	2.52	1.56	3.39
74.1 ± 1.3	3.78	1.56	3.26
72.4 ± 1.6	5.04	1.56	3.14

(d) cHo in Na^+ Binding Media (cont'd):

$I = 6.35 \times 10^{-3}$ M

K_{app}	$(Na^+) \times 10^4$ [M]	$[Fe(CN)_6^{4-}] \times 10^4$ [M]	$(T^+) \times 10^3$ [M]
90.2 ± 1.4	0	1.56	4.8
87.4 ± 1.6	1.24	1.56	4.67
85.0 ± 2.0	2.49	1.56	4.55
80.1 ± 0.6	3.74	1.56	4.43
78.9 ± 1.1	4.98	1.56	4.30

III. K_{app} in Cl^- Binding Media

(a) Cytochrome c:

$I = 3.94 \times 10^{-3}$ M

K_{app}	$(Cl^-) \times 10^4$ [M]	$[Fe(CN)_6^{4-}] \times 10^4$ [M]	$(T^+) \times 10^3$ [M]
68.6 ± 1.5	0	1.66	2.27
72.4 ± 1.5	1.93	1.66	2.27
76.9 ± 1.6	2.90	1.66	2.27
81.1 ± 2	3.86	1.66	2.27
86.8 ± 4	4.83	1.66	2.27

$I = 5.31 \times 10^{-3}$ M

K_{app}	$(Cl^-) \times 10^4$ [M]	$[Fe(CN)_6^{4-}] \times 10^4$ [M]	$(T^+) \times 10^3$ [M]
75.5 ± 2.2	0	1.66	3.64
82.5 ± 1.2	1.90	1.66	3.64
85.2 ± 2	2.86	1.66	3.64
89.2 ± 3	3.81	1.66	3.64
90.5 ± 2	9.76	1.66	3.64

$I = 6.19 \times 10^{-3}$ M

K_{app}	$(Cl^-) \times 10^4$ [M]	$[Fe(CN)_6^{4-}] \times 10^4$ [M]	$(T^+) \times 10^3$ [M]
90.6 ± 2.0	0	1.64	4.55
98.4 ± 3.4	1.88	1.64	4.55
101.0 ± 2.3	2.83	1.64	4.55
104.4 ± 1.1	3.77	1.64	4.55
107.5 ± 2.4	4.72	1.64	4.55

(b) Guanidinated Cytochrome c:

$I = 4.38 \times 10^{-3}$ M

K_{app}	$(Cl^-) \times 10^4$ [M]	$[Fe(CN)_6^{4-}] \times 10^4$ [M]	$(T^+) \times 10^3$ [M]
107.4 ± 2.4	0	1.65	2.73
112.3 ± 2.3	0.96	1.65	2.73
116.2 ± 2.6	1.92	1.65	2.73
115.0 ± 2.6	2.88	1.65	2.73
116.2 ± 2.0	3.84	1.65	2.73

$I = 5.27 \times 10^{-3}$ M

K_{app}	$(Cl^-) \times 10^4$ [M]	$[Fe(CN)_6^{4-}] \times 10^4$ [M]	$(T^+) \times 10^3$ [M]
114.9 ± 2.2	0	1.65	3.64
120.8 ± 2.1	0.951	1.65	3.64
124.3 ± 2.3	1.90	1.65	3.64
123.7 ± 2.5	2.85	1.65	3.64
124.3 ± 2.3	3.81	1.65	3.64

$I = 6.2 \times 10^{-3}$ M

K_{app}	$(Cl^-) \times 10^4$ [M]	$[Fe(CN)_6^{4-}] \times 10^4$ [M]	$(T^+) \times 10^3$ [M]
126.7 ± 1.8	0	1.65	4.55
129.3 ± 2.6	0.945	1.65	4.55
132.0 ± 2.6	1.89	1.65	4.55
132.0 ± 2.7	2.83	1.65	4.55
132.0 ± 2.6	3.78	1.65	4.55

(c) 1-TNP-Cytochrome c:

$I = 3.86 \times 10^{-3}$ M

K_{app}	$(Cl^-) \times 10^4$ [M]	$[Fe(CN)_6^{4-}] \times 10^4$ [M]	$(T^+) \times 10^3$ [M]
72.4 ± 2.3	0	1.61	2.27
79.9 ± 1.1	1.14	1.61	2.27
84.2 ± 1.1	2.27	1.61	2.27
86.9 ± 2.0	3.41	1.61	2.27
87.9 ± 2.7	4.55	1.61	2.27

(c) 1-TNP-Cytochrome c (cont'd):

$I = 5.13 \times 10^{-3}$ M

K_{app}	$(Cl^-) \times 10^4$ [M]	$[Fe(CN)_6^{4-}] \times 10^4$ [M]	$(T^+) \times 10^3$ [M]
87.3 ± 2.0	0	1.61	3.64
92.9 ± 2.3	1.12	1.61	3.64
96.4 ± 3.2	2.24	1.61	3.64
99.4 ± 3.5	3.48	1.61	3.64
99.4 ± 3.5	4.47	1.61	3.64

$I = 5.95 \times 10^{-3}$ M

K_{app}	$(Cl^-) \times 10^4$ [M]	$[Fe(CN)_6^{4-}] \times 10^4$ [M]	$(T^+) \times 10^3$ [M]
90.7 ± 2.2	0	1.61	4.55
94.8 ± 1.8	1.11	1.61	4.55
99.1 ± 2.3	2.21	1.61	4.55
102.4 ± 3.3	3.32	1.61	4.55
103.3 ± 2.9	4.43	1.61	4.55