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STUDIES ON CHEMICALLY MODIFIED CYTOCHROME c.

Part I. The Trinitrophenylated Cytochrome c.

By Keishiro WADA

## STUDIES ON CHEMICALLY MODIFIED CYTOCHROME c.

### Part I. The Trinitrophenylated Cytochrome c.

When bovine cytochrome c was trinitrophenylated with trinitrobenzene sulfonate (TNBS) under the specific conditions, the trinitrophenylated (TNP-) cytochrome c preparations, in which 1- to 5-TNP-groups were incorporated into the  $\epsilon$ -amino groups of lysine residues, were obtained in homogeneous state. Several properties of the TNP-cytochrome c were examined as compared with those of the acetylated (1) and native cytochrome c. It was found that the enzymatic activity of TNP-cytochrome c in the cytochrome oxidase [E.C. 1.9.3.1] reaction was less than half of that of native one, even when only one TNP-group was incorporated into cytochrome c molecule.

From the amino acid analyses of these preparations of TNP-cytochrome c, it was found that the 13th lysine residue from the amino terminus of the protein was most reactive with TNBS and the 22nd lysine residue secondly reactive. The remarkable loss in activity of TNP-cytochrome c in the cytochrome oxidase reaction was discussed as compared with

the case of acetyl-cytochrome c.

Cytochrome c is very basic protein (pI = 10.8) and a total of lysine (18), histidine (3), and arginine (2) residues occupies more than 20 % of all the constituent amino acids 104 residues in bovine cytochrome c.

It has been reported that the biological and the physicochemical properties of cytochrome c are altered by the chemical modifications of  $\epsilon$ -amino groups of lysine residues of the molecule with the various reagents (2-5). Cytochrome c loses partially or completely the biological function with acetylation or succinylation, but guanidination does scarcely affect the function. We have attempted another chemical modification (trinitrophenylation) of lysine residues in cytochrome c molecule in order to obtain new information about the contribution of  $\epsilon$ -amino groups of lysine residues to the biological function of the protein. Trinitrophenylation of amino acids was already reported by Hirayama (6) in 1909, before dinitrophenylation by Abderhalden *et al.* (7). Trinitrobenzene sulfonate (TNBS) has been used by Okuyama and Satake (8) as a reagent of

the chemical modification to amino groups in protein. The reagent is soluble in water and reacts specifically with  $\alpha$ - and  $\epsilon$ -amino groups in protein.

It has been found that one trinitrophenyl (TNP) group is incorporated into a certain  $\epsilon$ -amino group of the protein molecule, when cytochrome c is modified by TNBS under the specific conditions, and that the modified cytochrome c preparation thus obtained (1-TNP-cytochrome c) possesses less than 50 % activity of native cytochrome c in the cytochrome oxidase reaction. On the other hand, as reported in the previous paper (1), 1-acetyl-cytochrome c, in which one acetyl group was incorporated into 22nd lysine residue of cytochrome c molecule, is as almost active as native protein. It seems interesting to investigate the difference in the action between 1-TNP-cytochrome c and 1-acetyl-cytochrome c in the cytochrome oxidase reaction. In the present paper, several properties of the TNP-cytochrome c preparations and the lysine residue which is the most reactive with TNBS have been reported.

#### MATERIALS AND METHODS

Cytochrome c was prepared from bovine heart muscle by the method of Hagihara et al. (9). The amount of cyto-

chrome c was determined spectrophotometrically using the extinction coefficient at 550 m $\mu$  of the reduced form, 27.7 mM<sup>-1</sup>.cm<sup>-1</sup>.

Cytochrome a [E. C. 1.9.3.1] was prepared from bovine heart muscle according to the method of Okunuki et al.

(10). The amount of cytochrome a was determined spectrophotometrically using the difference extinction coefficient between 605 m $\mu$  and 630 m $\mu$  of the reduced form, 16.5 mM<sup>-1</sup>.cm<sup>-1</sup>.

Proteolytic enzymes: Twice crystallized trypsin (E.C. 3.4.4.4) was purchased from Worthington Biochemical Corporation. Thermolysin isolated from Bacillus thermoproteolyticus was kindly supplied by Daiwa Kasei Co. Osaka, and used after four crystallization. Specific activity of this enzyme was 7736 PU/mg.

2, 4, 6-Trinitrobenzene sulfonate (TNBS) was purchased from Wako Pure Chemical Industries, Ltd.

Trinitrophenylated cytochrome c: TNBS (5 molar excess to free  $\epsilon$ -amino group of lysine) was added to cytochrome c dissolved in 0.1 M phosphate buffer (pH 7.4). After incubation for an hour at 0°C, the reaction mixture was dialyzed against 0.01 M phosphate buffer (pH 7.4) for 12 hr. To remove native cytochrome c, the dialyzate was treated by an Amberlite IRC-50 column. Cytochrome c which had been trinitrophenylated to a considerable extent was not adsorbed on the column,

while native protein was adsorbed. To obtain a homogeneous preparation, the unadsorbed solution was further chromatographed on a column of DEAE-cellulose. In the case of the TNP-cytochrome c prepared as described above, more than three moles of TNP groups per mole were bound to the protein. When TNBS was added at a ratio of 1 mole per mole of free  $\epsilon$ -amino group of lysine in cytochrome c molecule and the reaction was performed for 15 or 30 min, TNP-cytochrome c preparations which contained one to two TNP-groups in the protein molecule were obtained in chromatographically homogeneous state. Using the extinction coefficient at 345 m $\mu$ , 14.5 mM<sup>-1</sup>.cm<sup>-1</sup>, the number of  $\epsilon$ -mono-TNP-lysine residues in the modified cytochrome c preparations were calculated according to the following equation.

$$N = \frac{A_{345\text{m}\mu}^{\text{OX}} - 0.8 \times A_{550\text{m}\mu}^{\text{red}}}{A_{550\text{m}\mu}^{\text{red}}} \times \frac{27.7}{14.5}$$

Assay of cytochrome oxidase activity was carried out spectrophotometrically as described in the previous paper (1).

Measurement of oxidation-reduction potentials of TNP-cytochrome c: Oxidation-reduction potentials were measured according to the method of Davenport and Hill (11) with ferri-ferrocyanide system. Thirty  $\mu$ moles of cytochrome

c was dissolved in 2 ml of 0.05 M sodium phosphate buffer (pH 7.0) containing 1.5 mmoles of ferrocyanide, and then the resulting solution was titrated with an appropriate amount of ferricyanide. The amounts of reduced and oxidized forms of cytochrome c in the solution were calculated by the equation described in the previous paper (1), and were corrected for the volume change by the addition of ferricyanide solution. Normal oxidation-reduction potential ( $E'_0$ ) of the modified cytochrome c was determined assuming the  $E'_0$  value of ferri-ferrocyanide system to be 0.430 volt at 20°C, pH 4 - 10 (12).

Paper electrophoresis and measurement of the intrinsic viscosity and digestion of cytochrome c by thermolysin were performed as reported previously (1).

Removal of heme group from heme peptide: Removal of heme from heme peptide were carried out according to the method of Ambler (13) with the slight modification as described previously (1).

Digestion of cytochrome c by Nagarse (bacterial proteinase): After equilibrated at 30°C, 20 ml of cytochrome c solution (0.13 %) and 10 ml of Nagarse solution (0.015 % in 0.05 M phosphate buffer, pH 7.4) were mixed. Then, 3 ml of the reaction mixture was withdrawn after a definite period and blown into 2 ml of 1.25 M trichloroacetic acid



solution. The resulting suspension was allowed to stand for 30 min or more at 40°C and then filtered through filter paper. One ml of the filtrate thus obtained was used to determine the amount of acid soluble peptides according to the Folin's method. In order to calculate the degree of digestion, the value obtained without TCA precipitation was taken as the control.

Amino acid analysis: The purified TNP-peptides were hydrolyzed in twice distilled 5.7 N HCl for 20 - 24 hr at 110°C. All analyses were performed by the method of Spackman et al. (14) using Beckman Amino Acid Analyzer Model 120B with the accelerated system.

## RESULTS

### 1. Trinitrophenylation of cytochrome c.

When cytochrome c was treated for an hour with TNBS of five molar excess to  $\epsilon$ -amino group of lysine residue, the resulting modified protein preparation was not adsorbed on the column of Amberlite IRC-50 equilibrated with 0.1 M ammonium phosphate buffer (pH 7.2), while native cytochrome c was adsorbed on the column under the experimental conditions. When the preparation unadsorbed on the cation exchanger column was subjected to the chromatography by a DEAE-cellulose

column, three fractions were obtained; 3-, 4- and 5-TNP-cytochrome c preparations. When cytochrome c incubated with an equimolar amount of TNBS for 15 min was chromatographed by the Amberlite IRC-50 column equilibrated with 0.1 M ammonium phosphate buffer, the unadsorbed fraction was less than 2 % (See Table I) of the initially used amount of the protein, and three bands observed on the column.

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Table I

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About 40 % (the fraction corresponding to peak 4 in Fig. 1) of the protein in the reaction mixture consisted of cytochrome c without any TNP-group (zero-TNP-cytochrome c).

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Fig. 1

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This fraction showed the same  $R_f$  value as native cytochrome c on Amberlite IRC-50 column chromatography and did not show the spectral change as compared with native cytochrome c both in visible and ultraviolet regions. And the remaining

fractions peaks 2 and 1 were one- and two-TNP-cytochrome c, respectively. Peak 3 (minor component) also consisted of one-TNP-cytochrome c, suggesting that the compound was possibly an isomer of that included in peak 2.

When cytochrome c was reacted with TNBS for 30 min, about 30 % of the reaction mixture was composed of the molecular species which were not adsorbed on the Amberlite IRC-50 in the first chromatography. It was evident from Table I that the proportion of fraction adsorbed on the column to the unadsorbed fraction decreased with the reaction period.

## 2. Properties of TNP-cytochrome c

Spectral properties of TNP-cytochrome c:  $\epsilon$ -Mono-TNP-lysine shows an absorption maximum at 345 m $\mu$  and a shoulder around 420 m $\mu$ . With ferri-TNP-cytochrome c, changes in the absorption spectrum were observed in the region of shorter wavelengths than 500 m $\mu$ . Fig. 2 illustrates the absorption spectra of TNP-cytochrome c preparations.

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Fig. 2

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An appreciable change of absorption spectrum was observed in the region from 330 m $\mu$  to 380 m $\mu$ . The spectral change in this region was evidently due to the incorporation of TNP-groups into cytochrome c molecule. Therefore, it was found that an absorbance around 350 m $\mu$  increased linearly with increase of the TNP-group incorporated and the peak of the absorption band shifted slightly to shorter wavelengths, and the absorbance of Soret band increased. These spectral changes had been also observed by Okuyama and Shino (15) during progressive trinitrophenylation of hemoglobin. Their results were very similar to the present results with TNP-cytochrome c. No change was observed in the ultraviolet region shorter than 300 m $\mu$  with the trinitrophenylation of cytochrome c.

The extinction coefficients of TNP-ferrocycytochrome c preparations at 550 m $\mu$  were not altered on the assumption that there was no difference in the extinction coefficients at 550 m $\mu$  of the pyridine hemochromes of the chemically modified and native cytochrome c. The ratio of the absorbance at 550 m $\mu$  of ferrocycytochrome c to that of the pyridine hemochrome did not vary with the TNP-cycytochrome c preparations. It was concluded from the above results that the extinction coefficients of 550 m $\mu$  of TNP-ferrocycytochrome c preparations were the same values as that of native cytochrome c.

This was also confirmed by a relation of native cytochrome c to TNP-cytochrome c in the absorbance of the pyridine hemochrome on the molar basis as calculated from amino acid analyses.

Structural properties of TNP-cytochrome c.

When the  $\epsilon$ -amino groups of lysine residues in cytochrome c are chemically modified, the conformation of the protein molecule is expected to be altered in some fashion. Among the many methods to investigate the conformational change in protein molecule, the viscometric measurement and the digestion by Nagarse (16) were used in the present study.

The intrinsic viscosities of 1- and 2-TNP-cytochrome c preparations were smaller than that of native one, as observed with guanidinated cytochrome c. While 1- and 2-TNP-ferricytochrome c preparations were slowly digested by Nagarse like native ferricytochrome c. However, the TNP-cytochrome c preparations which had five or more TNP-groups per molecule were slightly soluble in water and were digested as rapidly as the TCA-treated (17), the 18-succinylated (1) and the heat treated preparations of cytochrome c. It seemed from these results that the conformational changes in the TNP-cytochrome c molecule hardly occurred, so far as only a few lysine residues were modified.

Electrostatic properties of TNP-cytochrome c.

The TNP-cytochrome c preparations were subjected to paper electrophoresis to detect the changes in the electrostatic properties of cytochrome c caused by the trinitrophenylation. Fig. 3 shows paper electrophoretic diagrams of the TNP-cytochrome c preparations at pH 7.2.

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Fig. 3

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The electrophoretic mobility of these preparations to the cathode at this pH decreased with the degree of the modification. The preparation which contained three TNP- groups per molecule did not migrate to either direction at pH 7.2. When 4 and more  $\epsilon$ -amino groups of the protein molecule were trinitrophenylated, the modified cytochrome c migrated to the anode. Therefore, it was evident that the net positive charges of cytochrome c molecule were lost with the trinitrophenylation of the amino groups. Moreover, it was found that there was a linear relationship between the number of trinitrophenylated amino group and the distance in migration of the corresponding cytochrome c preparations, as observed with acetylated cytochrome c preparations (1).

Oxidation-reduction potentials of TNP-cytochrome c.

The oxidation-reduction potentials of TNP-cytochrome c preparations were measured at pH 7.0 according to the method of Davenport and Hill (11) with the ferri-ferrocyanide system. As shown in Fig. 4, plots of  $\log \left( \frac{\text{Ferrocyanide}}{\text{Ferricyanide}} \right)$  versus  $\log \left( \frac{\text{Ferrocycytochrome } \underline{c}}{\text{Ferricycycytochrome } \underline{c}} \right)$  showed linear.

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Fig. 4

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The normal oxidation-reduction potential  $E'_0$  of TNP-cytochrome c preparations was calculated to be about 255 mv from the values of  $\log \left( \frac{\text{Ferrocyanide}}{\text{Ferricyanide}} \right)$  at half reduction of the modified cytochrome c. Under the same experimental conditions, the  $E'_0$  value of the native cytochrome c preparation was found to be the same as that of the modified cytochrome c preparation. Therefore, it seemed from these results that  $E'_0$  of cytochrome c was not altered by trinitrophenylation of a few  $\epsilon$ -amino groups in the molecule.

### 3. Catalytic activity.

As reported previously (1), some modified cytochrome c preparations which contained many  $\epsilon$ -amino groups chemically

modified, except for guanidinated cytochrome c, can act as an electron donor to cytochrome a, but cannot interact with cytochrome a resulting in the loss of cytochrome oxidase activity. It has been found that when a few  $\epsilon$ -amino groups of cytochrome c molecule are acetylated, the cytochrome a-activating function of the modified cytochrome c decreases linearly and gradually with the number of the amino group acetylated.

With TNP-cytochrome c, the decrease of the cytochrome a-activating function was not in linear relation to the number of TNP-group incorporated into the protein molecule, as shown in Fig. 5 and Table II.

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Fig. 5

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Table II

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The modified cytochrome c preparation in which only one TNP-group was incorporated into the protein molecule had less than 50 % of the cytochrome a-activating function observed



with native cytochrome c unlike 1-acetyl-cytochrome c preparation and the activity decreased gradually with the modified cytochrome c preparations in which two or more TNP-groups were incorporated per molecule. The cytochrome a-activating function was completely lost with 5- or 6-TNP-cytochrome c preparation.

To clarify the mechanisms by which cytochrome c lost the activity with trinitrophenylation, the reactions with cytochrome a of native cytochrome c and the modified proteins were analyzed by the methods of Lineweaver and Burk. The reaction rates obtained with various concentration of native cytochrome c and TNP-cytochrome c preparations were plotted by the double reciprocal procedure. It was evident from the results shown in Fig. 6 that  $K_M$  value for 1-TNP-cytochrome c was about three times larger than that of native cytochrome c ( $6.6 \times 10^{-6}$  M) and the maximum reaction velocity of this modified preparations was lower than that of native one.

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Fig. 6

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Zero-TNP-cytochrome c (cytochrome c preparation which had no TNP-group although it had been treated with TNBS) possessed

the same catalytic activity as native cytochrome c.

The pH-activity curves of the TNP-cytochrome c preparations were investigated in citrate-phosphate buffer. The optimal pH in cytochrome oxidase reaction, as shown in Fig. 7, was about 5.2 for native cytochrome c, while that for the TNP-cytochrome c preparations seemed to shift slightly to lower pH values as the number of TNP-groups incorporated into the protein molecule increased.

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Fig. 7

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#### 4. Determination of the lysine residue trinitrophenylated.

The lysine residues trinitrophenylated at first or secondly during the chemical modification were determined with the purified preparations of TNP-cytochrome c. About three  $\mu$ moles of the modified cytochrome c were transferred into 4.0 ml of 0.04 M Tris-HCl buffer (pH 8.0) containing 0.002 M  $\text{CaCl}_2$ , and incubated with one mg of thermolysin. The digested cytochrome c solutions were lyophilized and chromatographed by the phospho-cellulose column equilibrated with 0.05 M acetate : pyridine buffer (pH 3.91) (18).

Some yellow TNP-peptides and heme peptide were eluted from the column by the stepwise elution from 0.05 M acetate : pyridine buffer (pH 3.91) to 0.45 M acetate : pyridine buffer (pH 5.40) (18). After the heme peptide was rechromatographed by the phospho-cellulose column equilibrated with the buffer used above, heme moiety was removed by the method described by Ambler (13) and then oxidized with performic acid prepared previously (19). When heme moiety was removed from the heme peptide the resulting peptide showed the yellow color due to TNP-lysine residue. It was not clear whether these heme peptides contained TNP-groups before the removal of heme group. Each yellow peptide containing TNP-group was further purified by paper chromatography (n-butanol : pyridine : acetic acid : water = 15 : 10 : 3 : 12 (v/v)) and/or the high voltage paper electrophoresis.

Table III shows the amino acid compositions and the yields of the TNP-peptides which was isolated from l-TNP-cytochrome c. In addition to the heme peptide containing TNP-group, there were three yellow peptides; Y-I, Y-II and Y-III.

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Table III

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Y-II and Y-III were N-terminal and 68th to 74th peptides, respectively. Y-I was not analyzed. The amino acid composition of the heme peptide was the same as that of the peptide which was isolated from 1-acetyl-cytochrome c (1) or isolated by Matsubara et al. (20) from the thermolytic digests of bovine native cytochrome c. The heme peptide was the main TNP-peptide which contained 38.4 % of the TNP-groups incorporated into the protein molecule and three lysine residues in addition to  $\epsilon$ -TNP-lysine residue. After removal of heme moiety it was digested by trypsin, and seven peptides were obtained as shown in Table IV and Fig. 8. The main TNP-peptide (YT-1) contained one  $\epsilon$ -TNP-lysine residue and was recognized to correspond to the peptide from the 9th isoleucine to the 22nd lysine residue.

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Table IV

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Fig. 8

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As the lysyl-cysteyl bond (Fig. 8) was not attacked by trypsin, it was decided that about 90 % of the TNP-group which

was found in the modified protein molecule was incorporated into the 13th lysine residue. The Y-III peptide was the minor TNP-peptide (See below).

When 2-TNP-cytochrome c was analyzed in the same way as 1-TNP-cytochrome c described above, seven yellow peptides and heme peptide were isolated (Table V). Three of the yellow peptides (2Y-I, 2Y-III-b and 2Y-V) were not analyzed. The main TNP-peptide (2YT-2) was obtained from the tryptic digests of the heme peptide from which heme moiety had been removed before the digestion. The TNP-peptide (2YT-2) contained two moles of TNP-groups per mole (Table VI and Fig. 9).

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Table V

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Table VI

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Fig. 9

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In the 2-TNP-cytochrome c preparation the 13th and the 22nd

lysine residues were mainly trinitrophenylated. However, it seemed that the 2-TNP-cytochrome c preparation was a mixture of the two molecular species; one species was trinitrophenylated both in the 13th and 22nd lysine residues; the other, both in the 13th and another lysine residues, respectively. Because, the peptide (2YT-3) in which only one TNP-group was incorporated and the 22nd lysine residue was not modified was found in a small amount.

It was concluded from these results that TNBS reacted with specific  $\epsilon$ -amino groups of the lysine residues in cytochrome c molecule. Namely, the 13th lysine residue was trinitrophenylated at first, and the 22nd was modified secondly, while the 72nd or 73rd, the 5th, 7th or 8th, and the 99th or 100th lysine residues were slightly modified with the reagent.

#### DISCUSSION

It has been found that the trinitrophenylation of lysine residues in cytochrome c molecule is also controllable by the reaction conditions under which the chemical modification of the protein is performed, as suggested in acetylation and succinylation (1). In the present investigation, several properties of TNP-cytochrome c have been examined.

It is reasonable that the trinitrophenylation of lysine residues which charge positively at neutral pH results in the gradual decrease of the basicity of cytochrome c with the increase of trinitrophenylated lysine residues (Fig. 3). Thus, some TNP-cytochrome c preparations are easily separated from native cytochrome c on an Amberlite IRC-50 as indicated in Fig. 1. The absorption spectra of TNP-cytochrome c differ from these of the native protein in the wavelength region below 500 m $\mu$  and this may be explained as the sum of the spectra due to cytochrome c and those to TNP-lysine.

It has been confirmed by the measurement of viscosity and the susceptibility to proteinases that the trinitrophenylation of a few amino groups in cytochrome c molecule hardly affects the conformation of cytochrome c molecule.

On the other hand, it is very interesting that trinitrophenylation of one lysine residue of cytochrome c results in loss of more than 50 % of the activity of native cytochrome c, unlike acetylation of the protein (1). Cytochrome a possessed  $K_m$  value for l-TNP-cytochrome c about three times larger than for native cytochrome c and the maximum reaction velocity in the cytochrome oxidase reaction with the modified protein is smaller than that with the native protein. Such a drastic change in the catalytic property of cytochrome c as described above has not been observed in any chemical

modification of  $\epsilon$ -amino groups of the lysine residues in the protein molecule other than trinitrophenylation. It has been elucidated that the 13th lysine residue of cytochrome c molecule is the most reactive with TNBS and the 22nd lysine residue secondly reactive, while their reactivity with acetic anhydride is reversed as reported previously (1).

We reported previously that the 72nd or 73rd lysine residue was the most reactive with TNBS (21). The result was qualitatively obtained from a portion of tryptic digests exclusive of heme peptide, because TNP-lysine residue could not be detected spectrophotometrically in heme peptide without removal of heme moiety. The previous result has to be corrected at the present time. However, the TNP-peptide containing the 72nd or 73rd lysine residue has been recovered at about 5 % yield in the present study.

The results obtained in the present study do not agree with the data reported by Sato et al. (22) about the reactive lysine residues in yeast cytochrome c. Namely, they have reported with yeast cytochrome c that  $\alpha$ -amino group of threonine residue in N-terminus is the most reactive with TNBS, and the 4th, the 89th and the 100th lysine residues are relatively reactive. In bovine cytochrome c, N-terminus is acetylated, and glutamic acid and glycine residues are substituted for the 4th and the 89th lysine residues, respective-



ly. However, the 4th and the 100th lysine residues in yeast cytochrome c appear to correspond to the 5th and the 99th or 100th lysine residues which are a little reactive with TNBS in bovine cytochrome c.

It may be assumed that the most reactive lysine residue toward TNBS is not identical with that toward acetic anhydride as a behavior of 1-TNP-cytochrome c in the cytochrome oxidase reaction is different from that of 1-acetyl-cytochrome c. If the 13th lysine residue, however, are essential for the cytochrome oxidase reaction, the radical loss of the activity should be observed with 2-acetyl-cytochrome c which has two modified lysine residues at the site of the 13th and the 22nd (1), being similar to 2-TNP-cytochrome c (Fig. 9). Nevertheless, 2-acetyl-cytochrome c is as 74 % active as native cytochrome c in the cytochrome oxidase reaction (1), although the 13th lysine residue is acetylated. Therefore, it seems probable that the enzymatic activity of cytochrome c is lost by trinitrophenylation, but not by acetylation, in the 13th lysine residue. As suggested in the previous paper (1), it appears that the radical loss of the activity of cytochrome c is caused when the tertiary structure around the 13th lysine residue in cytochrome c molecule is disturbed by the hydrophobicity or the bulkness of TNP-group. This seems to be quite reasonable assumption. The 13th lysine residue

neighbors on the cysteine residue to which heme is bound. According to Dickerson et al. (23), the 13th lysine residue resides across the top of the crevice of cytochrome c molecule. When the 13th lysine residue is modified by TNBS, the planely spreaded structure of TNP-group may partially cover the entrance to the crevice of the molecule. Thus, it is expected that cytochrome c loses the activity by trinitrophenylation although there is no appreciable change in the viscosity and the susceptibility to proteinase of the protein.

#### ACKNOWLEDGEMENT

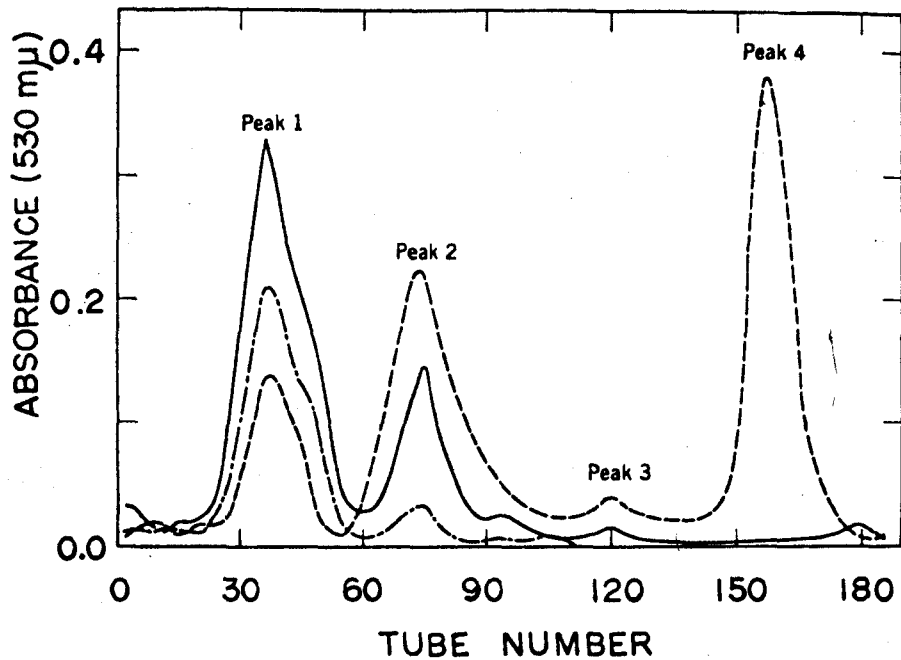
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#### REFERENCES

1. K. Wada and K. Okunuki; J. Biochem., 64, 667 (1968).
2. H. Fraenkel-Conrat, R. C. Bean and H. Lineweaver; J. Biol. Chem., 177, 365 (1949).
3. A. F. S. A. Habeeb, H. G. Cassidy and S. J. Singer; Biochim. Biophys. Acta, 29, 587 (1958).
4. C. M. Stevens and J. A. Bush; J. Biol. Chem., 183, 139 (1950).
5. S. Takemori, K. Wada, K. Ando, M. Hosokawa, I. Sekuzu and K. Okunuki; J. Biochem., 52, 28 (1962).
6. K. Hirayama, Z. physiol. Chem., 59, 290 (1909).
7. E. Abderhalden and P. Blumberg; Z. physiol. Chem., 65, 318 (1910).
8. T. Okuyama and K. Satake; J. Biochem., 47, 454 (1960).
9. B. Hagihara, K. Tagawa, I. Morikawa, M. Shin, and K. Okunuki; J. Biochem., 45, 725 (1958).
10. K. Okunuki, I. Sekuzu, T. Yonetani and S. Takemori; J. Biochem., 45, 847 (1958).
11. H. E. Davenport, and R. Hill; Proc. Roy. Soc., B, 139, 327 (1952).
12. W. M. Clark, B. Cohen and H. D. Gibbs; Publ. Health Rep., Washington, 40, 1131 (1925).
13. R. P. Ambler; Biochem. J., 89, 349 (1963).
14. D. H. Spackman, W. H. Sreine, and S. Moore; Anal. Chem., 30, 1190 (1958).

15. T. Okuyama, and H. Shino; cited in "Protein, Nucleic Acid, and Enzyme", Kyoritsu, Tokyo, 2, 355 (1959).
16. K. Okunuki; Adv. Enzym., ed. by F. F. Word, Interscience, New York, 23, 29 (1961).
17. M. Nozaki; J. Biochem., 47, 592 (1960).
18. P. E. Canfield and C. B. Anfinsen; J. Biol. Chem., 238, 2684 (1963).
19. D. H. W. Hirs; J. Biol. Chem., 219, 611 (1956).
20. H. Matsubara, A. Singer, R. Sasaki and T. H. Jukes; Biochem. Biophys. Res. Commun., 21, 242 (1965).
21. K. Okunuki, K. Wada, H. Matsubara and S. Takemori; "Oxidases and Related Redox Systems", ed. by T. E. King, H. S. Mason and M. Morrison; Wiley, New York, Vol. 2, 549 (1965).
22. C. Sato, K. Titani and K. Narita; J. Biochem., 60, 682 (1966).
23. R. E. Dickerson, M. L. Kopka, J. E. Weinzier, J. C. Venum, D. Eisenberg, and E. Margoliash; "Structure and Function in Cytochromes", ed. by K. Okunuki, M. D. Kamen and I. Sekuzu, Tokyo Univ. Press, Tokyo, p.225 (1968).

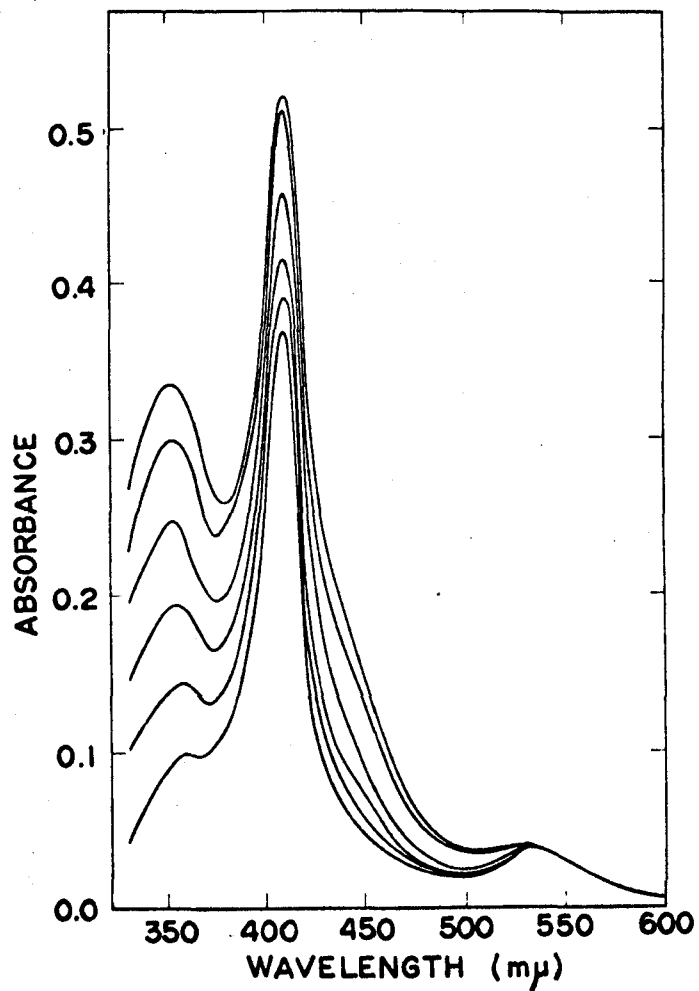
Fig. 1



Chromatogram of the TNP-cytochrome c preparations adsorbed on Amberlite IRC-50 column.

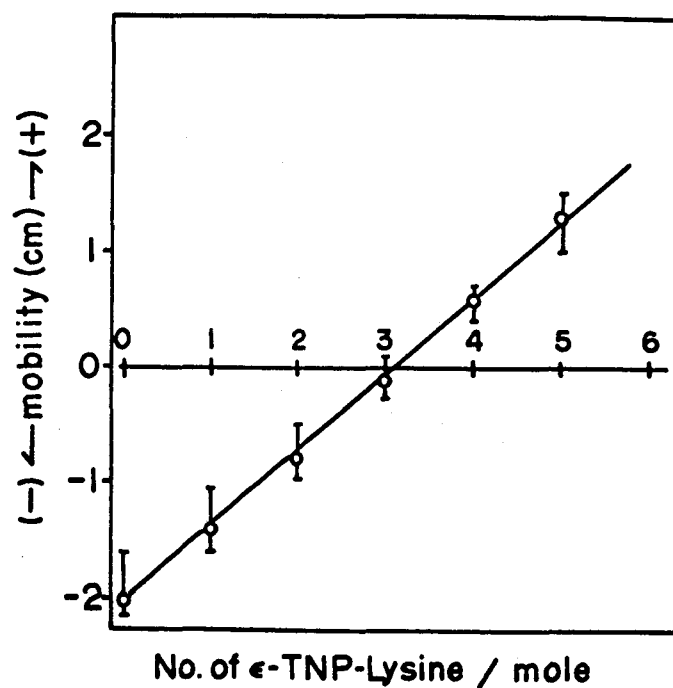
This chromatography was carried out using Amberlite IRC-50 column equilibrated with 0.2 M ammonium phosphate buffer (pH 7.4). The curves indicate the fractions obtained by different of reaction periods with TNBS. -----, 15 min reaction; \_\_\_\_\_, 30 min reaction; and -.-.-.-.-, 60 min reaction. (See Table I).

Fig. 2



Absorption spectra of TNP-cytochrome c preparations. Absorption spectra were measured with the oxidized forms of the preparations in 0.02 M sodium phosphate buffer (pH 7.2). The curves in the figure indicate , from the top to the bottom, the absorption spectra of 5-, 4-, 3-, 2- and 1-TNP-cytochrome c and native cytochrome c.

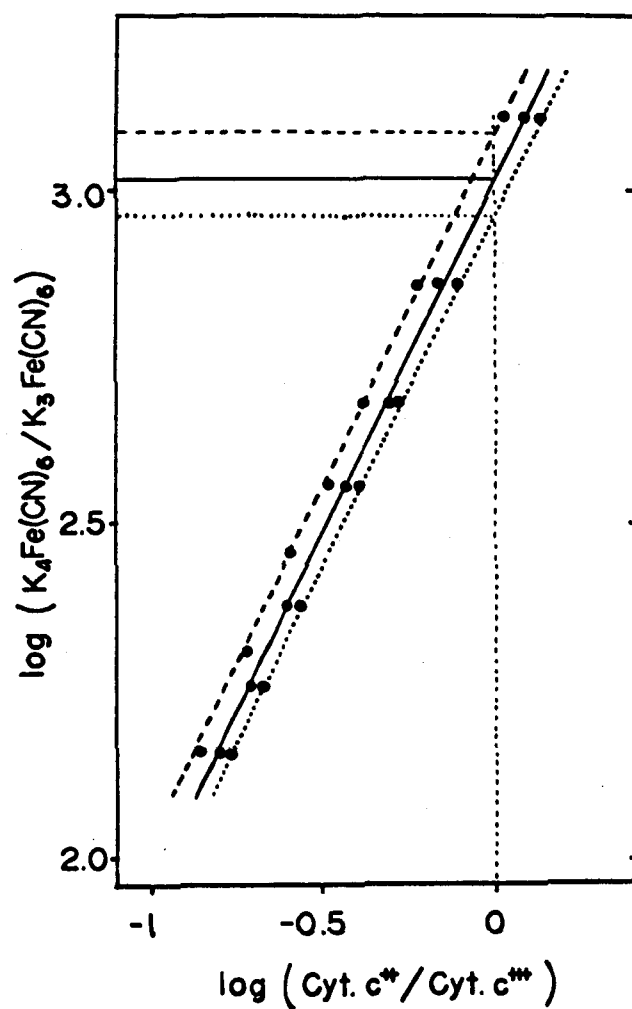
Fig. 3



Relationship between the number of amino groups trinitrophenylated and paper electrophoretic mobility (cm) in native cytochrome c and TNP-cytochrome c.

The electrophoresis was performed in 0.025 M potassium phosphate buffer at pH 7.2 for 5 hr using Toyo filter paper No. 51 which was pretreated with Quatamin 24P.

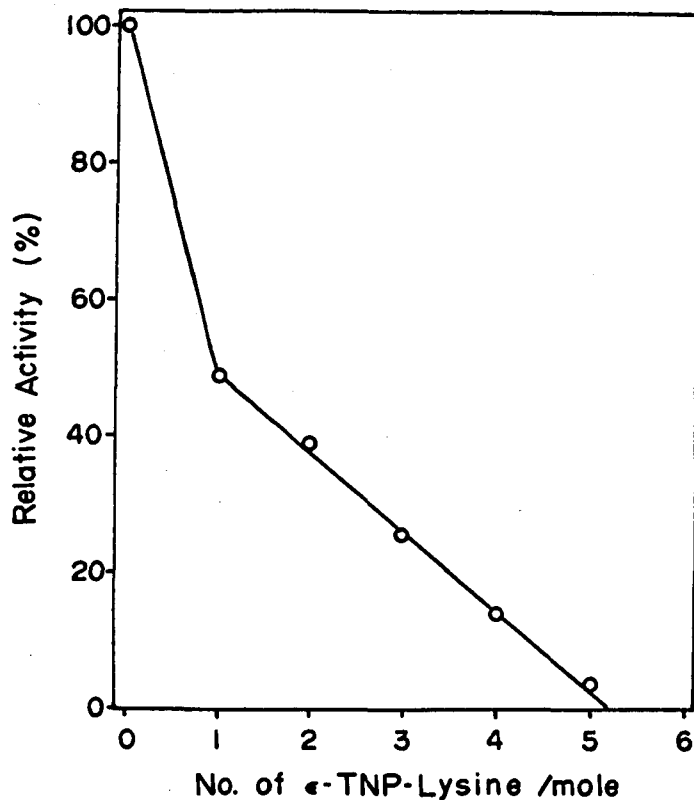
Fig. 4



Determination of normal oxidation-reduction potentials of native cytochrome c and TNP-cytochrome c with the ferri-ferrocyanide systems. ———, Native cytochrome c; ·······, 1-TNP-cytochrome c; - - - - - , 2-TNP-cytochrome c.



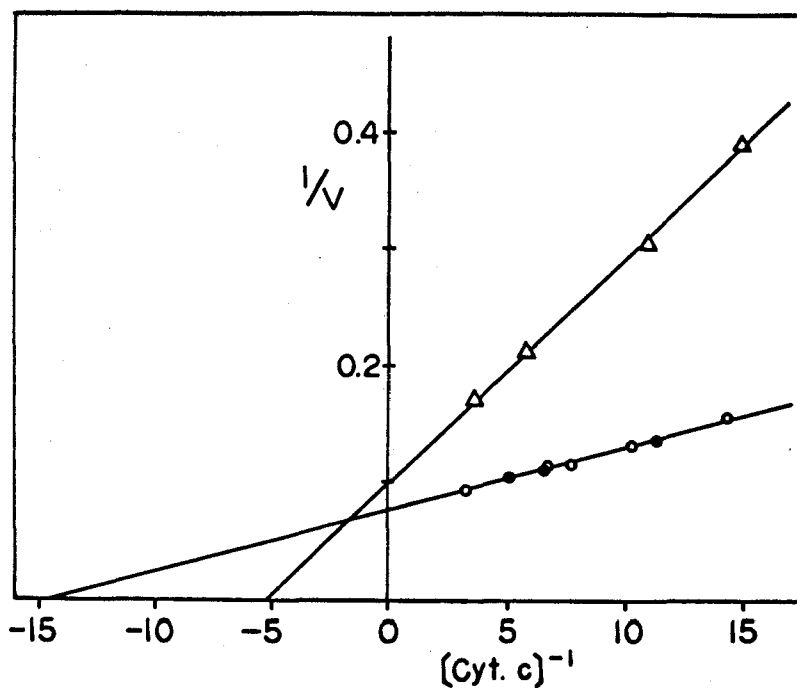
Fig. 5



Relationship between the number of trinitrophenylated  $\epsilon$ -amino group and the activity of TNP-cytochrome c in the cytochrome oxidase system.

Cytochrome oxidase activity was measured spectrophotometrically. The reaction mixture consisted of 15  $\mu$ M TNP- and native ferrocytochrome c, 0.05 M  $\text{Na}_2\text{HPO}_4$  and 0.025 M citric acid (pH 6.0). The reaction was initiated by the addition of 3  $\mu$ M cytochrome a to the reaction mixture (final 2.0 ml). Cytochrome oxidase activity was expressed in terms of the first order rate constant relative to that for native cytochrome c.

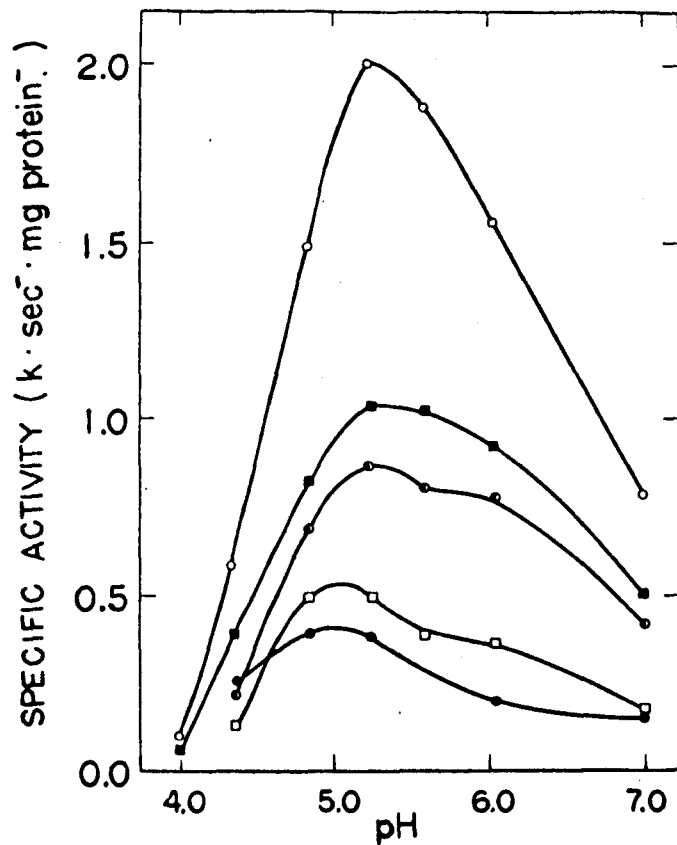
Fig. 6



The analysis of cytochrome oxidase reaction with TNP-cytochrome c by the method of Lineweaver and Burk.

The reaction conditions used were the same as for Fig. 5 except for the concentration of cytochrome c.  $\triangle$ , 1-TNP-cytochrome c;  $\bullet$ , 0-TNP-cytochrome c; and  $\circ$ , native cytochrome c.

Fig. 7



pH-activity curves in the cytochrome oxidase reaction with TNP-cytochrome c preparations.

Oxidation of the reduced cytochrome c by cytochrome a was followed spectrophotometrically at various pH values under the same conditions as in Fig. 5. —○—, Native cytochrome c; —■—, 1-TNP-cytochrome c; —●—, 2-TNP-cytochrome c; —□—, 3-TNP-cytochrome c and —●—, 4-TNP-cytochrome c.

Fig. 8 Amino acid sequences of the peptides isolated from the digests of 1-TNP-cytochrome c by thermolysin and trypsin.

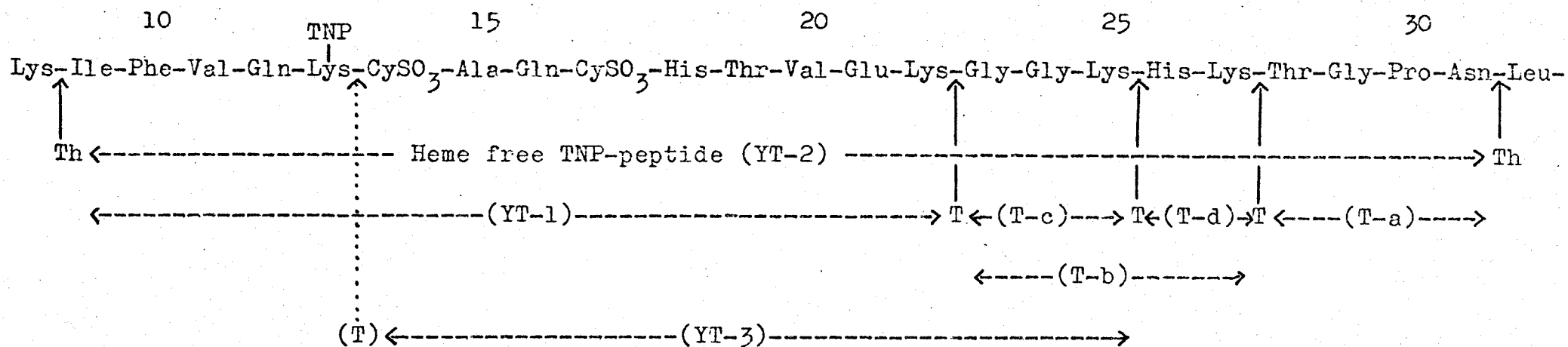


Fig. 9 Amino acid sequences of the peptides isolated from the digests of 2-TNP-cytochrome c by thermolysin and trypsin.

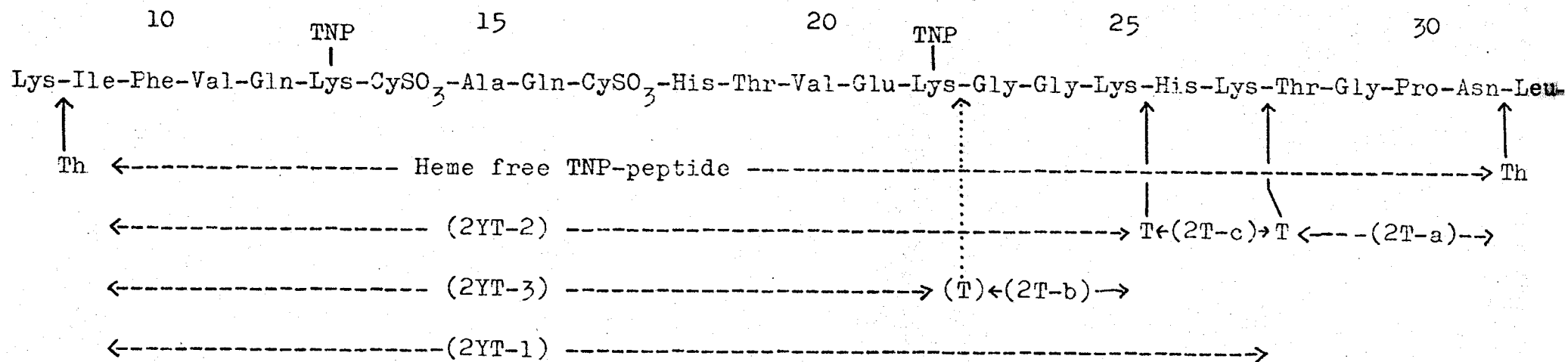


Table I TNP-cytochrome c preparations and their recoveries.

Reaction period (min)	Fraction	No. of TNP-lysine residues		Recovery (%)
		Expected	Found	
15	1	3	-	less than 2
	2	2	-	19.5
	3	1	0.96	40.0
	4	0	0.00	36.7
30	1*	4	4.23	10.3
	2	3	2.75	18.2
	3	2	1.96	45.4
	4	1	0.97	17.0
60	1*	4	4.17	21.6
	2	3	2.99	53.7
	3	2	2.14	23.9
	4	1	-	less than 1

\* Preparations contained TNP-cytochrome c which possessed 4 or more TNP-groups in the molecule.

Table II Enzymatic activity on chemically modified cytochrome c

Preparation	No. of masked lysine residues	Relative cytochrome oxidase activity (%) <sup>*</sup>	Time required for half reduction of cytochrome <u>a</u> (sec) <sup>**</sup>
Native cyt. <u>c</u>	0	100	15
0-TNP-	0	100	15
1-TNP-	1	50	25
2-TNP-	2	39	40
3-TNP-	3	23	-
4-TNP-	4	14	-
5-TNP-	5	3	-
<hr/>			
1-Acetyl- <sup>***</sup>	1	90	-
2-Acetyl- <sup>***</sup>	2	74	-
3-Acetyl- <sup>***</sup>	3	61	-
<hr/>			
4-Succinyl- <sup>***</sup>	4	0	225
<hr/>			
9-Guanid- <sup>***</sup>	8.8	110	-
13-Guanid- <sup>***</sup>	12.6	116	-
17-Guanid- <sup>***</sup>	16.6	123	-

\* Enzymatic activity of each preparation of the chemically modified cytochrome c was measured spectrophotometrically as described in the legend of Fig. 5.

\*\* The time required for half reduction of cytochrome a were measured by tracing the absorbance at 605 m $\mu$ . The assay system contained 0.1 M phosphate buffer (pH 7.4), 1 % Emasol 1130, 1 mM KCN, 30  $\mu$ M cytochrome a, 1 mM hydroquinone and 10  $\mu$ M chemically modified cytochrome c and native cytochrome c, in a total volume of 3.0 ml. The reaction was initiated by the addition of hydroquinone.

\*\*\* These data were cited from the previous paper of this series ( 1 ).

Table III Amino acid compositions of TNP-peptides and yields of the peptides from thermolytic digests of 1-TNP-cytochrome c.

Amino acids	Y-I	Y-II	Y-III	Heme peptide*
TNP-Lys**	+	0.92	1.02	0.23
Lys	-	2.06	1.40	3.27
His	-	-	-	1.80
Asp	-	1.00	1.08	1.18
Thr	-	-	0.10	2.09
Glu	-	1.06	1.08	3.26
Pro	-	-	0.88	0.92
Gly	-	1.98	0.30	2.94
Ala	-	-	-	1.02
Val	-	0.90	0.10	2.02
Ile	-	-	0.16	0.71
Leu	-	-	0.85	0.10
Tyr	-	-	0.72	-
Phe	-	-	-	0.77
Cysteic acid	-	-	-	1.67
<hr/>				
Yield*** ( $\mu$ moles)	0.003	0.031	0.150	2.65
(%)	0.1	1.0	5.0	88.4
<hr/>				
Site of peptide	-	1-8	68-74	9-31
<hr/>				
Probable site of TNP-Lys	-	5, 7, or 8	72 or 73	13, 22, 25 or 27

\* Each value was calculated after the removal of heme group except for TNP-lysine residue.

\*\* TNP-lysine residues were measured spectrophotometrically using the absorbance at 345 m $\mu$  before acid hydrolysis.

\*\*\* These values were calculated using the amount of the starting material as a standard.



Table IV Amino acid compositions of TNP-peptides and yields of the peptides from tryptic digests of the heme free TNP-peptide prepared from 1-TNP-cytochrome c

Amino acids	YT-1*	YT-2*	YT-3*	T-a*	T-b*	T-c*	T-d*
TNP-Lys**	0.90	0.93	1.13	-	-	-	-
Lys	1.26	3.46	1.25	-	1.98	1.00	1.11
His	0.84	1.62	0.77	-	0.62	-	0.88
Asp	-	1.20	-	0.99	-	-	-
Thr	1.04	2.01	1.15	1.12	-	-	-
Glu	3.26	3.42	2.43	-	-	-	-
Pro	-	0.89	-	0.67	-	-	-
Gly	0.32	2.99	2.40	1.24	2.38	2.40	-
Ala	1.01	1.11	1.15	-	-	-	-
Val	1.90	2.14	1.04	-	-	-	-
Ile	0.86	0.76	-	-	-	-	-
Phe	0.84	0.79	-	-	-	-	-
Cysteic acid	1.76	1.61	1.78	-	-	-	-
Yield*** (μmoles) (%)	2.0 66.7	0.3 10.0	0.02 0.7	2.2 73.3	0.2 6.7	2.0 66.7	1.8 60.0
Site of peptide	9-22	9-31	14-25	28-31	23-27	23-25	26-27
Probable site of TNP-Lys.	13	13, 22, 25 or 27	22	-	-	-	-

\* Each peptide was obtained from the heme free TNP-peptide by tryptic digestion at 35°C and pH 8.0 for 2 hr, and purified by paper chromatography and paper electrophoresis.

\*\* TNP-lysine residues were spectrophotometrically measured using the absorbance at 345 mμ before acid hydrolysis.

\*\*\* These values were calculated using the amount of the starting material as standard.

Table V Amino acid compositions of TNP-peptides and yields of the peptides from thermolytic digests of 2-TNP-cytochrome c.

Amino acids	2Y I	2Y II-a	2Y II-b	2Y III-a	2Y III-b	2Y IV	2Y V	Heme peptide *
TNP-Lys**	+	+	0.91	1.13	+	+	+	0.64
Lys	-	2.19	2.31	1.29	-	1.35	-	2.21
His	-	-	-	-	-	-	-	1.82
Asp	-	1.15	0.93	1.02	-	-	-	1.15
Thr	-	-	-	0.11	-	-	-	2.14
Glu	-	0.92	0.90	1.05	-	-	-	3.26
Pro	-	-	-	0.81	-	-	-	0.95
Gly	--	2.20	2.01	0.32	-	-	-	3.18
Ala	-	-	-	-	-	-	-	0.92
Val	-	0.92	0.85	-	-	-	-	1.91
Ile	-	0.64	-	-	-	-	-	0.70
Leu	-	-	-	0.85	-	1.00	-	0.18
Tyr	-	-	-	0.70	-	-	-	-
Phe	-	-	-	-	-	-	-	0.75
Cysteic acid	-	-	-	-	-	-	-	1.66
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Yield*** (%)	0.004 0.13	0.005 0.2	0.086 2.8	0.162 5.4	0.005 0.2	0.02 0.7	0.006 0.2	2.45 81.6
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Site of peptide	-	1-9	1-8	68-74	-	98-100	-	9-31
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Probable site of TNP-Lys	-	5, 7 or 8	5, 7 or 8	72 or 73	-	99 or 100	-	13, 22, 25 or 27

\* Each value was calculated after the removal of heme group except for TNP-lysine residues.

\*\* TNP-lysine residues were spectrophotometrically measured using the absorbance at 345 mμ before acid hydrolysis.

\*\*\* These values were calculated using the amount of the starting material as a standard.

Table VI Amino acid compositions of TNP-peptides and yields of the peptides from tryptic digests of heme free TNP-peptide of 2-TNP-cytochrome c.

Amino acids	2YT-1*	2YT-2*	2YT-3*	2T-a*	2T-b*	2T-c*
TNP-Lys**	1.88	1.97	0.99	-	-	-
Lys	2.46	1.35	1.29	-	++	1.28
His	1.92	0.96	0.87	-	-	0.73
Asp	-	-	-	0.98	-	-
Thr	0.83	0.85	0.92	1.11	-	-
Glu	3.19	3.20	3.22	-	-	-
Pro	-	-	-	0.69	-	-
Gly	2.43	2.45	0.46	1.24	+++	-
Ala	0.91	0.88	0.95	-	-	-
Val	1.34	1.82	1.90	-	-	-
Ile	0.72	0.71	0.72	-	-	-
Phe	0.70	0.73	0.69	-	-	-
Cysteic acid	1.65	1.69	1.71	-	-	-
Yield*** (μmoles) (%)	0.42 14.0	1.36 45.3	0.04 1.3	1.74 58.0	- -	1.40 46.6
Site of peptide	9-27	9-25	9-22	28-31	23-25	26-27
Probable site of TNP-Lys	13, 22 or 25	13 or 22	13	-	-	-

\*Each peptide was obtained from the heme free TNP-peptide by tryptic digestion at 35°C and pH 8.0 for 2 hr, and purified by paper chromatography and paper electrophoresis.

\*\*TNP-lysine residues were spectrophotometrically measured using the absorbance at 345 mμ before acid hydrolysis.

\*\*\*These values were calculated using the amount of the starting material as a standard.

STUDIES ON CHEMICALLY MODIFIED CYTOCHROME c.

Part II. The 22-acetyl-Cytochrome c.

By Keishiro WADA

STUDIES ON CHEMICALLY MODIFIED CYTOCHROME c.

Part II. The 22-Acetyl-Cytochrome c.

SUMMARY

When bovine cytochrome c was modified with a large excess of acetic anhydride, was obtained 22-acetyl-cytochrome c in which all of lysine (18 moles) and tyrosine (4 moles) residues were acetylated. The spectral changes in the oxidized form, the autoxidizability and the easiness in binding with the externally added ligands such as cyanide and carbon monoxide showed that the conformation around heme in 22-acetyl-cytochrome c might be appreciably disturbed.

The transition between the different spin states was observed with the acetylated cytochrome c in the pH region below 7. At room temperature, it occurred at pH 5.0, and at liquid nitrogen temperature below pH 1.0. It was found that the high spin state at room temperature in the pH region between 1.0 and 7.0 was converted to the low spin state at liquid nitrogen temperature, and that they made a thermally equilibrated mixture between -160°C and -70°C.

## INTRODUCTION

The chemical modifications of  $\epsilon$ -amino groups of lysine residues in cytochrome c have been performed by various reagents (1, 2), and are known to affect greatly the catalytic property of the protein in the cytochrome oxidase reaction (1). Nevertheless, they hardly affect the spectral property of cytochrome c, even when all  $\epsilon$ -amino groups of its lysine residues are acetylated (1). We have reported previously (1) that when four moles of tyrosine residues besides all  $\epsilon$ -amino groups of lysine residues are acetylated, the remarkable changes occur in the absorption spectrum of cytochrome c in the oxidized form but not in the reduced form, and the activity of the protein in the cytochrome oxidase reaction is completely lost. The modified cytochrome c in which 18 moles of lysine residues and 4 moles of tyrosine residues are acetylated (namely, 22-acetylcytochrome c) is very autoxidizable and combines easily with carbon monoxide. In an acidic solution, 22-acetylcytochrome c exhibits the high spin bands at 620 and 495  $\mu$  which are not observed with native ferricytochrome c, while the low spin band at 530  $\mu$  is not seen with the modified protein.

Theorell and Åkesson (3) have already reported that in a very acidic solution native cytochrome c in the

oxidized form is brown in color and that there are several types (type I-V) of native ferricytochrome c depending on the pH value; type I below pH 0.42, type II between pH 0.42 and 2.5, type III between pH 2.5 and 9.35, type IV between pH 9.35 and 12.76, and type V above pH 12.76.

The transition between the high and low spin states of 22-acetyl-ferricytochrome c seems to correspond to that between types III and II of native protein as reported by Theorell and Åkesson ( 3 ). Therefore, the conformation around heme group in 22-acetyl-cytochrome c seems to be similar to that of native ferricytochrome c dissolved in the very acidic solution.

In the present paper, some properties of 22-acetyl-cytochrome c, i.e. pH dependency of the autoxidizability, reactivity with CO and low temperature spectrum are reported. We also describe that not only tyrosine residue but also methionine and tryptophan residues seem to contribute to the conformation around the heme group.

#### MATERIALS AND METHODS

The 22-acetyl-cytochrome c in which 22-acetyl groups per mole of cytochrome c were incorporated was prepared as

follows: Bovine cytochrome c which was purified by Hagihara's method ( 4 ) (10 mg/ml) was dissolved in 10 mM sodium phosphate buffer (pH 7.2) and the solution was incubated with the acetic anhydride 20-fold molar excess to  $\epsilon$ -amino groups of lysine residues at 0°C. During the reaction, the pH of the solution was kept at 7.2 with addition of 1 N NaOH. After the consumption of alkali had ceased, the reaction mixture was treated with a Sephadex G-25 column to separate the modified protein from acetate. The acetyl-cytochrome c thus obtained possessed 4.2 moles of O-acetyl-tyrosine residues, but had no  $\epsilon$ -amino group which was detectable by Habeeb's method ( 5 ). O-Acetyl-tyrosine residues were determined by the method of Simpson et al. ( 6 ).

The autoxidation of the modified cytochrome c were measured with Cary spectrophotometer Model 15 at 21°C. The reaction mixture was composed of 12  $\mu$ M ferrocytochrome c or modified ferrocytochrome c, 10 mM sodium phosphate buffer and 300  $\mu$ M EDTA. One volume of 48  $\mu$ M ferrocytochrome c solution was mixed with three volumes of 13  $\mu$ M sodium phosphate buffer containing EDTA, and the absorption spectra around 550 m $\mu$  of the mixture were immediately traced after pH of the solution was adjusted to a definite value by HCl. The recording of the spectra was performed repeatedly with a definite interval. After the determination of the spectra



was completed, pH of the reaction mixture was measured. The autoxidations in various pH's were compared with one another on the basis of the first order rate constant.

The ferrocyclochrome c was prepared by reduction of ferricyclochrome c with addition of a small amount of sodium dithionite. To remove excess dithionite, the resulting ferrocyclochrome c solution was treated with a Sephadex G-25 column equilibrated with 300  $\mu$ M sodium phosphate buffer (pH 7.4) containing 300  $\mu$ M EDTA.

CO-complexes of 22-acetyl-cyclochrome c were prepared by bubbling of CO for 10 min through the modified ferrocyclochrome c solution in various pH's.

The absorption spectra at low temperature were measured with Cary spectrophotometer, Model 14 fitted with the low temperature spectrophotometry apparatus derived by Kawai ( 7 ). The solution of oxidized cyclochrome c pH of which was adjusted to a definite value with diluted  $H_3PO_4$  was poured into the cell of 2 mm light path, and gradually frozen by immersing in liquid nitrogen.

The pH values were determined with Horiba's combination pH electrode, 6028-10T.

The measurement of magnetic susceptibility ( 8 ) were carried out with a magnetic torsion balance in the temperature range from liquid nitrogen temperature to about 0°C.

The lyophilized cytochrome c preparations (330 mg) were dissolved in about 1 ml of 0.05 M  $\text{NaH}_2\text{PO}_4$ . After adjusting pH 3.0 with  $\text{H}_3\text{PO}_4$ , the cytochrome c solution was transferred to the sample capsule which held the solution of approx. 0.7 ml and rapidly frozen by immersing in liquid nitrogen. Then the sample capsule was set in the magnetic torsion balance, and the susceptibility of the sample was plotted automatically and continuously as a function of temperature on an X-Y recorder. The temperature was measured by the use of a thermocouple of Au-Co alloy and Pt.

## RESULTS

### Absorption spectrum of 22-acetyl-cytochrome c

As reported previously ( 1 ), the absorption spectrum of 22-acetyl-ferricytochrome c at pH 7.0 was different from that of the native protein in the several points. With the modified cytochrome c, (1) the low spin band at 530  $\mu$  which was observed with native cytochrome c was lowered, (2) the high spin bands at 620 and 495  $\mu$  appeared, and (3) the absorbance at the Soret band increased and that at 270-280  $\mu$  decreased except for a small peak at 290  $\mu$  which remained constant. At pH 4.0, the considerably clear appearance

of the high spin bands was observed. Such a spectrum as described above was very similar to the absorption spectrum of the carboxymethylated cytochrome c (9,10) and to that of the high spin type ferrihemoproteins; e.g. horse radish peroxidase, cytochrome c peroxidase, catalase and acidic ferri-hemoglobin and ferri-myoglobin.

As shown in Fig.1, the absorption spectrum of the 22-acetyl-cytochrome c preparation varied with pH in the oxidized form of the protein.

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Fig. 1

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The presence of isosbestic points at 660, 583, 508 and 485 m $\mu$  among the absorption spectra at different pH values indicates the existence of the two components which contribute to the appearance of the high and low spin bands. Nevertheless, the absorption spectra of the reduced form did not change above pH 4.0 and showed the typical ferrohemochrome spectra which could not be distinguished from that of native ferrocytochrome c except that the extinction coefficient at 550 m $\mu$  slightly decreased (Table I).

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Table I

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Autoxidation of 22-acetyl-cytochrome c.

Although the 22-acetyl-ferrocyclochrome c was little autoxidizable at neutral pH values, its autoxidizability depended on pH as shown in Fig. 2.

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Fig. 2

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Native cytochrome c was hardly autoxidizable at neutral pH values but slightly reactive with oxygen of acidic pH values. The autoxidation of the modified preparation was very fast in pH range lower than neutral pH value as compared with that of native ferrocyclochrome c. As Fig. 2 shows, the autoxidation process of 22-acetyl-ferrocyclochrome c was not monophasic at pH 6.1, 5.0 and 4.5. This fact suggests that reaction system has contained two components; one is responsible for the fast autoxidation and the other the slow reaction.

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Table II

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Table II shows the first order rate constants of the fast and slow autoxidations at various pH values. They have been calculated from the results shown in Fig.2. When the amount of each component was qualitatively estimated from the curves shown in Fig. 2, it was found that the component which was responsible for the fast autoxidation increased with lowering pH. In pH 4.0, most part of the preparation in the reaction system seemed to be composed of the fast autoxidizable component. The pH-dependent transition between the fastly and slowly autoxidizable components seemed to run parallel with that between the high spin and low spin bands in spectrophotometry (Fig. 1).

Effects of external ligands on absorption spectrum.

The effects of externally added ligands such as cyanide, azide, imidazole and pyridine on the absorption spectrum of 22-acetyl-cytochrome c were examined at a neutral pH. The effects of cyanide and azide on the absorption spectrum of native cytochrome c had been studied by Horecker and Kornberg ( 11 ) and Horecker and Stannerd ( 12 ), respectively. We studied also the effects of the externally added ligands on native cytochrome c and extended their experiments. When large excesses (0.5 M) of cyanide and azide were added to native cytochrome c solution, the absorption spectrum

characteristic of cyanide- and azide-complexes of ferricytochrome c were respectively obtained, and the cyanide-ferricytochrome c complex was found to be considerably stable. Namely, a mixture which consisted of 10  $\mu\text{M}$  cytochrome c and 0.5  $\text{M}$  cyanide was incubated for 60 min at room temperature and was treated with the Sephadex G-25 column equilibrated with 0.05  $\text{M}$  sodium phosphate buffer (pH 7.0). The cytochrome c solution thus obtained still showed the absorption spectrum of the cyanide-complex which possessed a broad band at 530  $\text{m}\mu$  and the Soret band at 412  $\text{m}\mu$  in the oxidized form. When the cyanide-complex was reduced by dithionite, the absorption bands appeared at 555, 526 and 419  $\text{m}\mu$  and they immediately shifted to the positions of the  $\alpha$ ,  $\beta$  and Soret bands, respectively, of native ferrocytochrome c (Fig. 3). However, the presence of cyanide, azide or imidazole equimolar to cytochrome c, or of 2 % pyridine did not affect the spectrum of native cytochrome c.

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Fig. 3

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When 22-acetyl-cytochrome c, however, the high spin bands of the oxidized form were immediately replaced with two bands of the cyanide-complex on addition of equimolar amount of cyanide at pH 7.0 and the reduction of the resulting complex

by dithionite proceeded in the same way as the complex of native cytochrome c (Fig. 3). Furthermore, when imidazole equimolar to the modified cytochrome c and 2 % pyridine were added, the 22-acetyl-ferricytochrome c lost the high spin bands and became spectrally undistinguishable from the native protein except that the extinction of the Soret band was larger than that of the native protein.

No appreciable effect of azide was observed on the absorption spectrum of the modified cytochrome c so far as tested.

#### CO-complex formation of 22-acetyl-cytochrome c.

As described above, the absorption spectra of 22-acetyl-ferrocycytochrome c at various pH values were similar to that of native preparation. However, as shown in Fig. 4, the 22-acetyl-ferrocycytochrome c preparation was found to be mixture of two components; one combined with CO and the other was insensitive to the reagent. The CO-binding component increased with lowering of pH value.

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Fig. 4

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The absorption spectrum of the CO-complex was quite similar to that of native preparation which was measured by Butt and Keilin ( 15 ) at an alkaline pH; it possessed two broad

peaks at 560 and 532 m $\mu$ , a very sharp Soret peak at 414 m $\mu$  and a small peak at 395 m $\mu$ . As the spectrum of the modified cytochrome c under CO scarcely changed in the pH range below 4.5, the amount of CO-binding component was calculated at various pH values on the assumption that the preparation was completely bound with CO in this pH range.

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Table III

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Table III shows the rate of the CO-complex in the modified cytochrome c preparation at various pH values. From the figures in the table, it is clear that the CO-complex formation with the preparation depends on pH. This runs parallel to the pH-dependent transition between fastly and slowly autoxidizable components as described above.

Temperature-dependent spectral change.

As judged from the absorption spectrum, the 22-acetyl-ferricytochrome c preparation mostly seemed to be consisted of the high spin species at pH 4.0 and at room temperature (Fig. 1). Yonetani et al. ( 14 ) have reported the temperature-dependent transition of the spin states of cytochrome c peroxidase on the basis of the low temperature spectrophotometry. Therefore, the temperature dependency of the absorption spectra of 22-acetyl-ferricytochrome c was investigated.



When the solution of the modified cytochrome c preparation at pH 4.0 was frozen in liquid nitrogen temperature, brown color of the solution due to the high spin bands immediately changed into red. Thus, the absorbance of the low spin band at 530 m $\mu$  increased remarkably in the absorption spectrum at liquid nitrogen temperature, while that of the high spin bands at 620 and 495 m $\mu$  decreased considerably. However, the high spin bands were still seen at this temperature (Fig. 5).

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Fig. 5

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When the temperature of the modified cytochrome c was gradually elevated from liquid nitrogen temperature to about  $-70^{\circ}\text{C}$ , the low spin component decreased, and the high spin component increased. The temperature-dependent transition between the high spin and low spin components was completely reversible. However, the isosbestic points among the absorption spectra at various temperatures was not detected unlike in the pH-dependent spectral change as shown in Fig. 1. From these results it was expected that 22-acetyl-ferricytochrome c at pH 4.0 might be in thermal equilibrium between the high spin and low spin states. Therefore, the relationship between pH and the spin state was examined also

at liquid nitrogen temperature. With the modified cytochrome c, the high spin bands began to appear below pH 2.0, and the spin state transition occurred at about pH 1.0 (Fig. 6), whereas the high spin bands began to appear below pH 1.0 with native cytochrome c. Temperature-dependent transitions of spin states were not detected at the extremely acidic pH values in both native cytochrome c and 22-acetyl-ferri-cytochrome c. These spin states observed above with the modified cytochrome c seemed to correspond to the different types (types I, II and III) reported by Theorell and Åkesson ( 3 ).

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Fig. 6

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Magnetic susceptibility of native cytochrome c in acidic pH.

The paramagnetic susceptibility of native ferricytochrome c was measured at varied temperatures. The pH of the solution was adjusted to 3.0 at room temperature. At this pH, a part of native cytochrome c was in thermal equilibrium between the high spin and low spin states as judged from the low temperature spectrophotometry.

The magnetic susceptibilities at various temperatures of native ferricytochrome c were shown in Fig. 7.

It was found that native ferricytochrome c at pH 3.0 was pure

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Fig. 7

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low spin compound with an effective Bohr magneton number ( $n_{\text{eff}}$ ) of 2.02 between  $-160^{\circ}\text{C}$  and  $-196^{\circ}\text{C}$ . The  $n_{\text{eff}}$  value was identical with the value at  $22^{\circ}\text{C}$  reported by Theorell ( 15 ). However, above  $-160^{\circ}\text{C}$ , the native cytochrome c preparation became a thermally equilibrated mixture of the high spin and low spin states and its magnetic susceptibility deviated gradually from the Curie law as the temperature rised. Acetylation of tyrosine residues.

It was very interesting that the remarkable changes in the absorption spectra described above was not observed with 18-acetyl-cytochrome c preparation in which 18 moles of acetyl groups were incorporated per mole of the protein ( 1 ). Therefore, it seemed that the acetylation of tyrosine residues of cytochrome c might be responsible for such a remarkable spectral change as observed with 22-acetyl-cytochrome c. To clarify this, the acetylation of lysine and tyrosine residues were performed by stepwise addition of acetic anhydride to cytochrome c solution under the conditions as used to prepare 22-acetyl-cytochrome c. As Table IV shows, the acetylation of tyrosine residues occurred after all lysine residues had been acetylated.

With 18-succinyl-cytochrome c ( 1 ) in which 18 moles

of succinyl groups were incorporated per mole of the protein, (but tyrosine residues do not react with succinic anhydride ( 16 )), the pH-dependent spectral change as observed with 22-acetyl-cytochrome c was not detected. However, 18-succinyl-4-acetyl-cytochrome c preparation which was obtained by the treatment of 18-succinyl-cytochrome c preparation with acetic anhydride showed the pH-dependent spectral change above pH 4.5 (below this pH value, the preparation was little soluble) as 22-acetyl-cytochrome c did.

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Table IV

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DISCUSSION

The absorption spectrum of native cytochrome c is stable in a wide range of pH and temperature ( 13 ) and is not affected in the presence of considerably concentrated denaturants ( 17 ). The spectral change is hardly caused even by acetylation and succinylation of all  $\epsilon$ -amino groups of lysine residues ( 1 ). The catalytic property of cytochrome c in the cytochrome oxidase reaction is considerably affected by the modifications by various reagents of a few  $\epsilon$ -amino groups of the protein molecule. It has been suggested by the X-ray analysis of the ferricytochrome c crystal ( 18 ) and by the solvent perturbation ( 19 ) that the heme group

is located in a crevice of the protein molecule and fixed by the central coordination of specific groups of the amino acid residues and by the hydrogen bondings between two propionic side chains of the heme and tyrosyl side chains situated at the bottom of the crevice, besides by the thioether bondings. Thus, it is reasonable to consider that the remarkable spectral change of 22-acetyl-cytochrome c in the oxidized form and the easy binding of externally added ligands to the heme iron of the protein results from the modification of tyrosine residues which are buried in the interior region of molecule before the modification. The conformation around the heme group in 22-acetyl-cytochrome c seems to be considerably disturbed. The speculation mentioned above will be further discussed as compared with the behavior of native cytochrome c in the acidic solution.

As shown in Fig. 6, the transition of spin states in native cytochrome c is strongly influenced by pH of the sample. It is reasonable to consider that the heme iron of cytochrome c in type III is coordinated by two ligands with the strong ligand field from the both sides of the heme, because cytochrome c shows the hemochrome spectrum in a wide range of pH and temperature and does not easily bind with the externally added ligands. However, Lumry and Sullivan

( 20 ) have suggested that one side (6th coordination position) of the heme is bound more weakly to the ligand from the protein molecule than the other side.

In type II, such a weakly bound ligand as supposed in type III seems to be replaced by water molecule or by a ligand with the weak ligand field from the protein molecule according to the conformational change of the protein moiety. It seems also likely that the protonation makes weak the bond between the heme iron and the ligand group. This compound, type II, exists in a thermal equilibrium between the high spin and low spin states according to the Boltzman distribution as has been experimentally proved by George et al. ( 21 ) (Fig. 5). Namely, it exists as the high spin state at room temperature and as the low spin state at liquid nitrogen temperature. In the present investigation, we have not analyzed in detail the results about the thermal equilibrium which have been obtained by the measurements of magnetic susceptibility at various temperatures, because the spin state at the low temperature in the type II was indistinguishable from that in the type III by the spectrophotometry. However, two low spin states in the types II and III seems not to be identical in regard to the electronic structure of the heme iron. This point should be elucidated by a future investigation.

Cytochrome c of the type II changes to the type I by further lowering of pH value (Fig. 6). Cytochrome c of the type I exists as the high spin state even at low temperature and exhibits no thermal equilibrium. The both ligand may be split off from the heme iron by the entrance of two protons as suggested by Boeri et al. (22). It seems probable that the interaction of the heme with protein moiety of cytochrome c is extremely disturbed by the acid denaturation of the protein. However, the transition from the type III to the type II and that from type II to type I are completely reversible.

Such a relationship as mentioned above between pH and the different types of cytochrome c is also observed with the 22-acetyl-cytochrome c. With native cytochrome c the transition points at room and liquid nitrogen temperatures have been found at pH 2.5 and pH 0.42, respectively, whereas they have shifted to pH 5.0 and pH 1.0, respectively, with the modified cytochrome c (Fig. 6). The shift of transition points to the upper pH values indicates that cytochrome c molecule becomes more unstable by the chemical modification than the original protein molecule. Consequently, the conformation around the heme moiety is easily affected by the change of pH and the ligand to the heme iron is easily replaced by the externally added ligands.

Another important fact is that such a conformational change around the heme moiety as caused by the chemical modification of cytochrome c affects the absorption spectrum in the oxidized form but not in the reduced form. As ferrocycytochrome c is not so easily digested by proteinases as ferricytochrome c ( 23 ), the former is thought to be more rigid in the structure than the latter. Furthermore, ferrocycytochrome c does not exhibit the temperature-effect and pH-effect in the ORD spectra unlike ferricytochrome c ( 24 ). These facts suggest that the ligand at the 6th coordination position in cytochrome c molecule is bound to the heme iron more strongly in the reduced form than in the oxidized form. Therefore, the type II of cytochrome c is indistinguishable from the type III in the absorption spectra of the reduced form. However, it is very interesting that existence of the two molecular species mentioned above are clearly observed with the modified ferrocycytochrome c in regard to the autoxidizability and the CO-complex formation (Figs. 2 and 4). This fact suggests that there is difference in the strength of the ligand coordination in ferrocycytochrome c molecule between the types II and III.

The remarkable spectral change in the acidic pH region as observed with 22-acetyl-ferricytochrome c in the present study is also detected with carboxymethylated ferricytochrome



c in which the 80th methionine residue is modified ( 9, 10 ), and with N-bromosuccinimide-treated cytochrome c in which the 59th tryptaphan residue is oxidized ( 25 ). Therefore, it seems likely that not only tyrosine residues but also the 80th methionine and the 59th tryptophan residues are important for the maintenance of the conformation around the heme moiety of cytochrome c.

Fanger et al. ( 26 ) have suggested on the basis of study on carboxymethylated Pseudomonas cytochrome c that the 61st methionine residue is one ligand to the heme iron of the protein, and that it is probable that the 80th methionine residue coordinates to the heme iron in the mammalian-type cytochrome c. It has been reported ( 27 ) that the thio-ether group of methionine functions a ligand to the heme iron resulting in the hemochrome spectra. However, the possibility that another ligand of cytochrome c molecule coordinates to the heme iron can not be roled out, as the hemochrome spectrum formation is not completely inhibited by the carboxymethylation of the 80th methionine residue in the mammalian-type cytochrome c, although the possibility that the resulting modified methionine residue can coordinate to the heme iron still remains.

The fact that the modification of amino acid residues which are located in the interior region of cytochrome c

molecule results in the remarkable spectral changes and the loss of function of the protein, may lead us to the conclusion that these residues contribute to the maintenance of the conformation around the heme moiety or that in the crevice structure.

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#### REFERENCES

1. K. Wada and K. Okunuki; J. Biochem., 64, 667 (1968).
2. K. Wada; Ann. Rep. Sci. Works, Fac. Sci., Osaka Univ.,  
12, 19 (1964).
3. H. Theorell and Å. Åkesson; J. Am. Chem. Soc., 63, 1812  
(1941).
4. B. Hagihara, K. Tagawa, I. Morikawa, M. Shin and K. Okunuki;  
J. Biochem., 45, 725 (1958).
5. A. F. S. A. Habeeb; Anal. Biochem., 14, 328 (1966).
6. R. T. Simpson, J. F. Riordan and B. L. Vallee; Biochemi-  
stry, 2, 616 (1963).
7. K. Kawai; Anal. Biochem., 26, 264 (1968).
8. T. Iizuka and M. Kotani; Biochim. Biophys. Acta, 154, 417  
(1968).
9. A. Schejter and P. George; Nature, 206, 1150 (1965).
10. K. Ando, H. Matsubara and K. Okunuki; Biochim. Biophys.  
Acta, 118, 240 (1966).
11. B. L. Horecker and A. Kornberg; J. Biol. Chem., 165, 11  
(1946).
12. B. L. Horecker and J. N. Stannard; J. Biol. Chem., 172,  
589 (1948).
13. W.D. Butt and D. Keilin; Proc. Roy. Soc., B.156, 429 (1962).
14. T. Yonetani, D. F. Wilson and B. Seamounts; J. Biol. Chem.,  
241, 5347 (1966).

15. H. Theorell; J. Am. Chem. Soc., 63, 1820 (1941).
16. J. F. Riordan and B. L. Vallee; Biochemistry, 3, 1768 (1964).
17. T. Yamanaka, T. Horio and K. Okunuki; J. Biochem., 51, 426 (1962).
18. R. E. Dickerson, M. L. Kopka, J. E. Weinzier, J. C. Venum, D. Eisenberg and E. Margoliash; "Structure and Function in Cytochromes", ed. by K. Okunuki, M. D. Kamen and I. Sekuzu, Univ. of Tokyo Press, Tokyo, p.225 (1968).
19. E. Stellwagen; J. Biol. Chem., 242, 602 (1967).
20. R. Lumry and J. Sullivan; Federation Proc., Suppl. 13, 22, 586 (1963).
21. P. George, J. Beetlestone and J. S. Griffith; "Hematin Enzymes", ed. by J. E. Falk, R. Lemberg and R. K. Morton, Pergamon, New York, p.105 (1961).
22. E. Boeri, A. Ehrenberg, K. G. Paul and H. Theorell; Biochim. Biophys. Acta, 12, 273 (1953).
23. K. Okunuki; Adv. Enzymol., ed. by F. F. Nord, Interscience, New York, 23, 29 (1961).
24. D. W. Urry; Proc. Natl. Acad. Sci., U. S., 54, 641 (1965).
25. T. Yonetani; "Structure and Function in Cytochromes", ed. by K. Okunuki, M. D. Kamen and I. Sekuzu, Univ. of Tokyo Press, Tokyo, p.289 (1968).
26. M. W. Fanger, T. P. Hettlinger and H. A. Harbury; Biochemistry, 6, 713 (1967).

27. H. A. Harbury, J. R. Cronin, M. W. Fanger, T. P. Hettlinger,  
A. J. Murphy, Y. P. Myer and S. N. Vinogradov; Proc. Natl.  
Acad. Sci., U. S., 54, 1658 (1965).

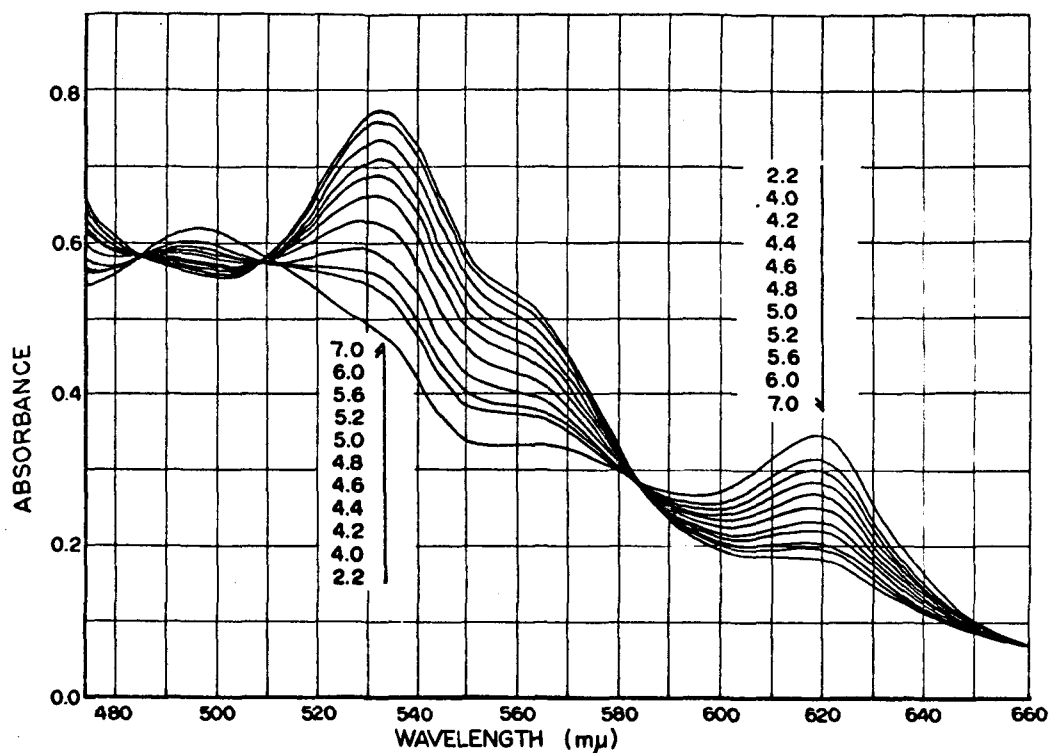


Fig. 1 Effect of pH on the visible absorption spectrum of 22-acetyl-ferricytochrome c

The sample was dissolved in 0.05 M sodium phosphate buffer (pH 7.4) and pH of the resulting solution was adjusted a definite value with HCl. After the measurement, pH was checked by the combination pH electrode. The numbers in the figure indicate the pH value at which the respective spectra were determined.

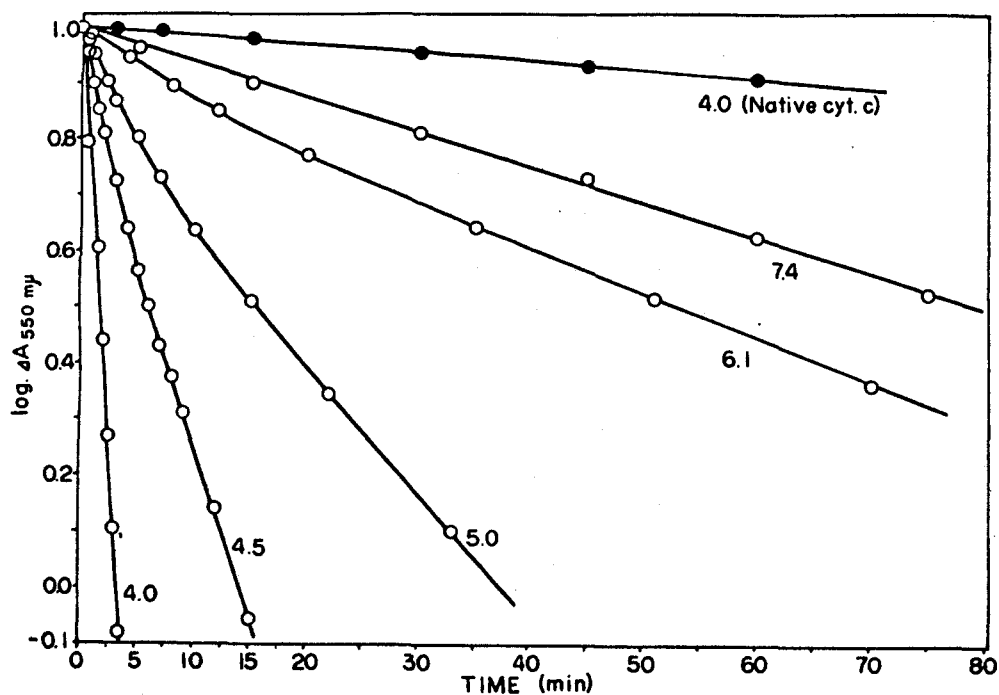


Fig. 2 Autoxidation of 22-acetyl-ferrocytochrome c at various pH values.

After the modified ferrocytochrome c solutions were diluted with 13  $\mu\text{M}$  sodium phosphate buffer containing 400  $\mu\text{M}$  EDTA and pH of the resulting solution was adjusted a definite value with HCl, the decreases in the absorbance at 550  $\mu\text{m}$  were measured. —●—, Native ferrocytochrome c (pH 4.0); —○—, 22-Acetyl-ferrocytochrome c. The numbers in the figure indicate the pH value at which the respective autoxidation were measured.

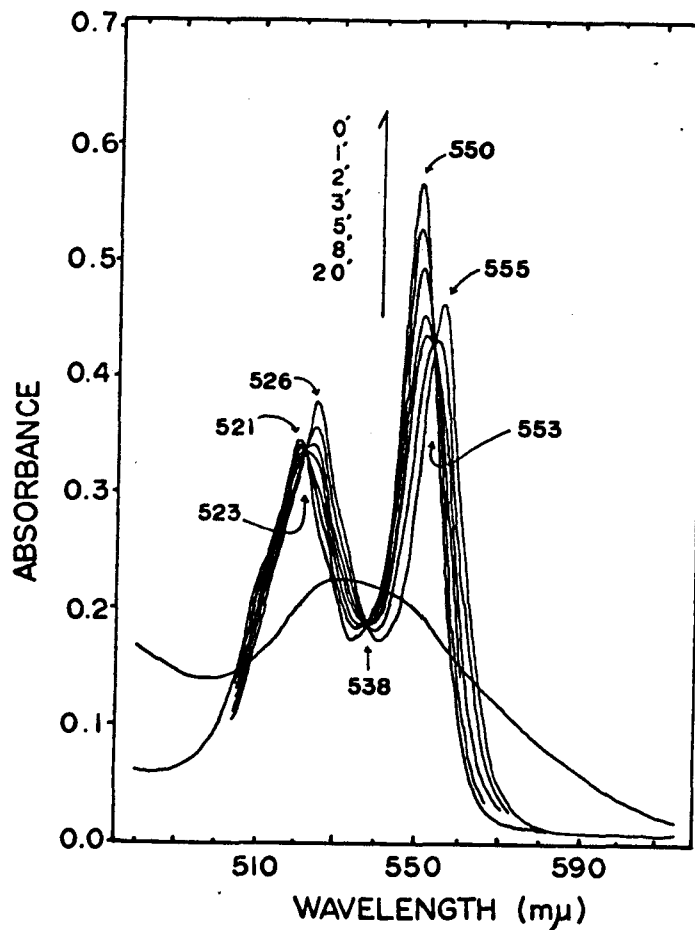


Fig. 3 Reduction process of the CN-complex of native cytochrome c.

Native cytochrome c in 0.05 M sodium phosphate buffer (pH 7.4) was incubated with 0.1 M KCN for one hour. The pH of the KCN solution had been adjusted to 7.4 before use. After the CN-complex spectrum of the oxidized protein was measured, a small amount of sodium dithionite was added to the cytochrome c solution. The absorption spectra of the CN-complex were recorded at times as indicated in the figure after the reduction of cytochrome c.



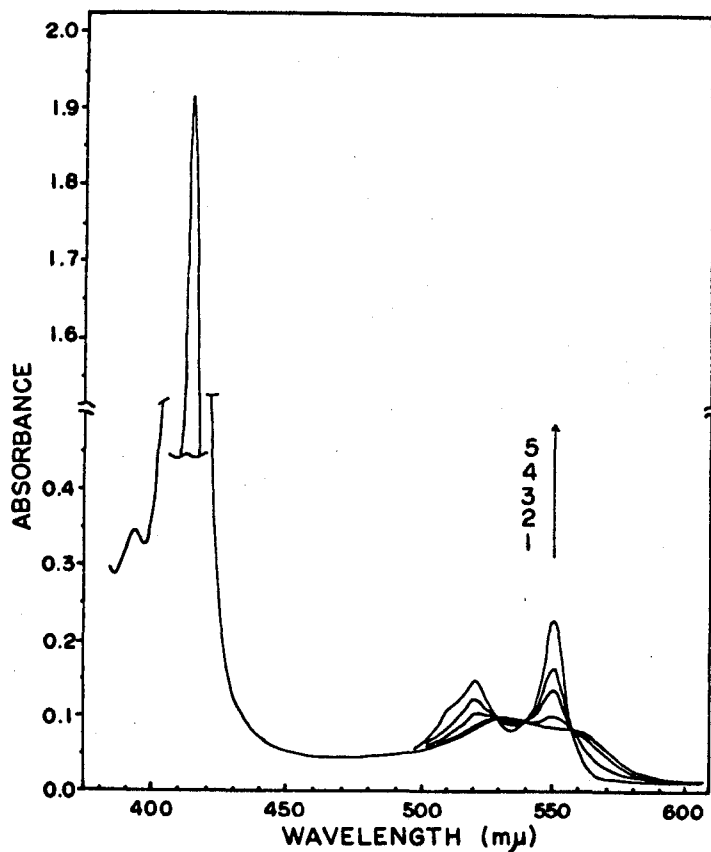


Fig. 4 Effect of pH on the CO-complex formation of 22-acetyl-ferrocyclochrome c.

The absorption spectra were measured after the modified ferrocyclochrome c solutions at various pH values were bubbled with CO for 10 min. Curve 1, at pH 4.0 and pH 4.5; curves 2-4, at pH 5.1, pH 6.0 and pH 7.4; curve 5, the spectrum at different pH values before CO-bubbling.

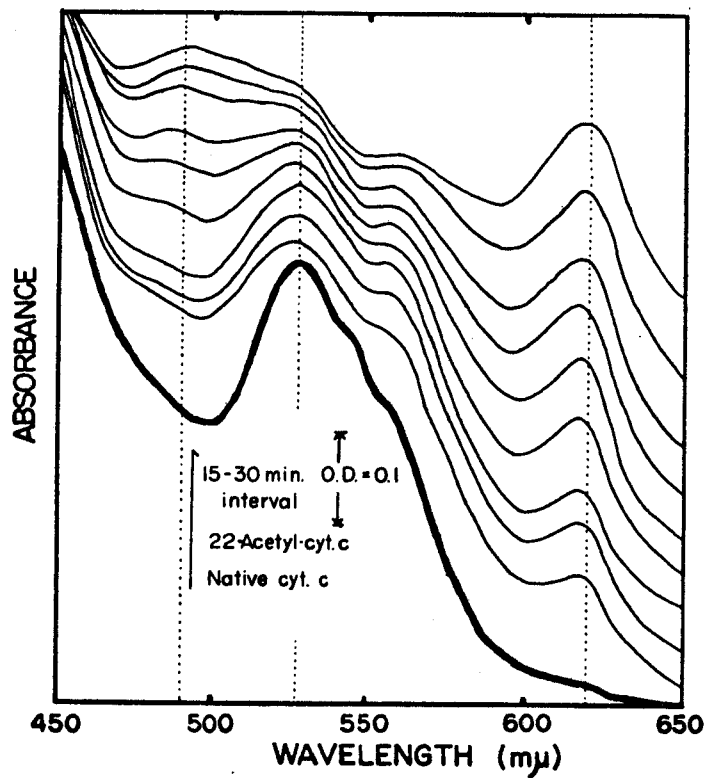


Fig. 5 Effect of temperature on the spectrum of 22-acetyl-ferricytochrome c.

The visible spectra of 22-acetyl-ferricytochrome c (pH 4.0) were traced several times while the temperature rised from liquid nitrogen temperature (the thin bottom line) to about  $-70^{\circ}\text{C}$  (the thin top line). The thick line shows the visible spectrum of native ferricytochrome c at liquid nitrogen temperature.

### RELATIONSHIP BETWEEN pH AND SPIN STATES

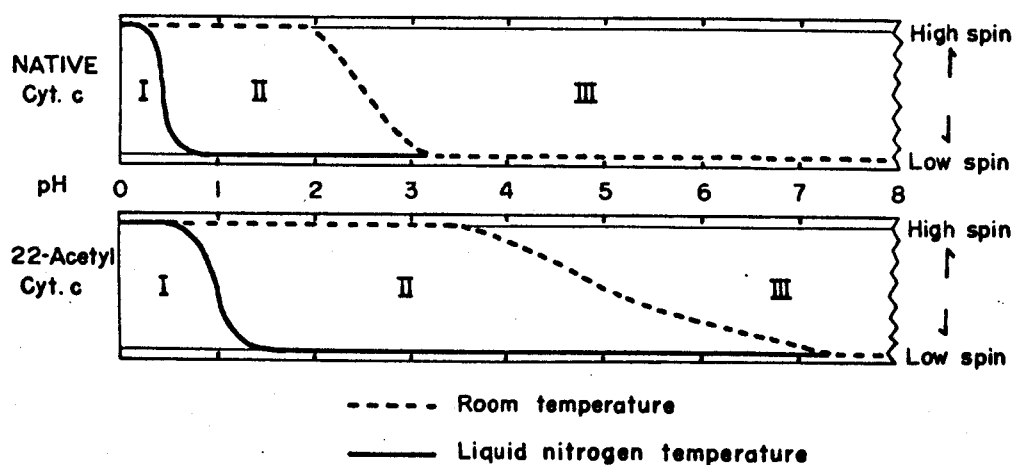


Fig. 6 Diagrams of relationship between pH and the spin states of native and the modified cytochrome c.

The ratio of the high spin to the low spin was qualitatively estimated by the method of Theorell and Åkesson ( 3 ) with the slight modifications. The transition of the spin states at room temperature was indicated by ----- and that at liquid nitrogen temperature by \_\_\_\_\_ .

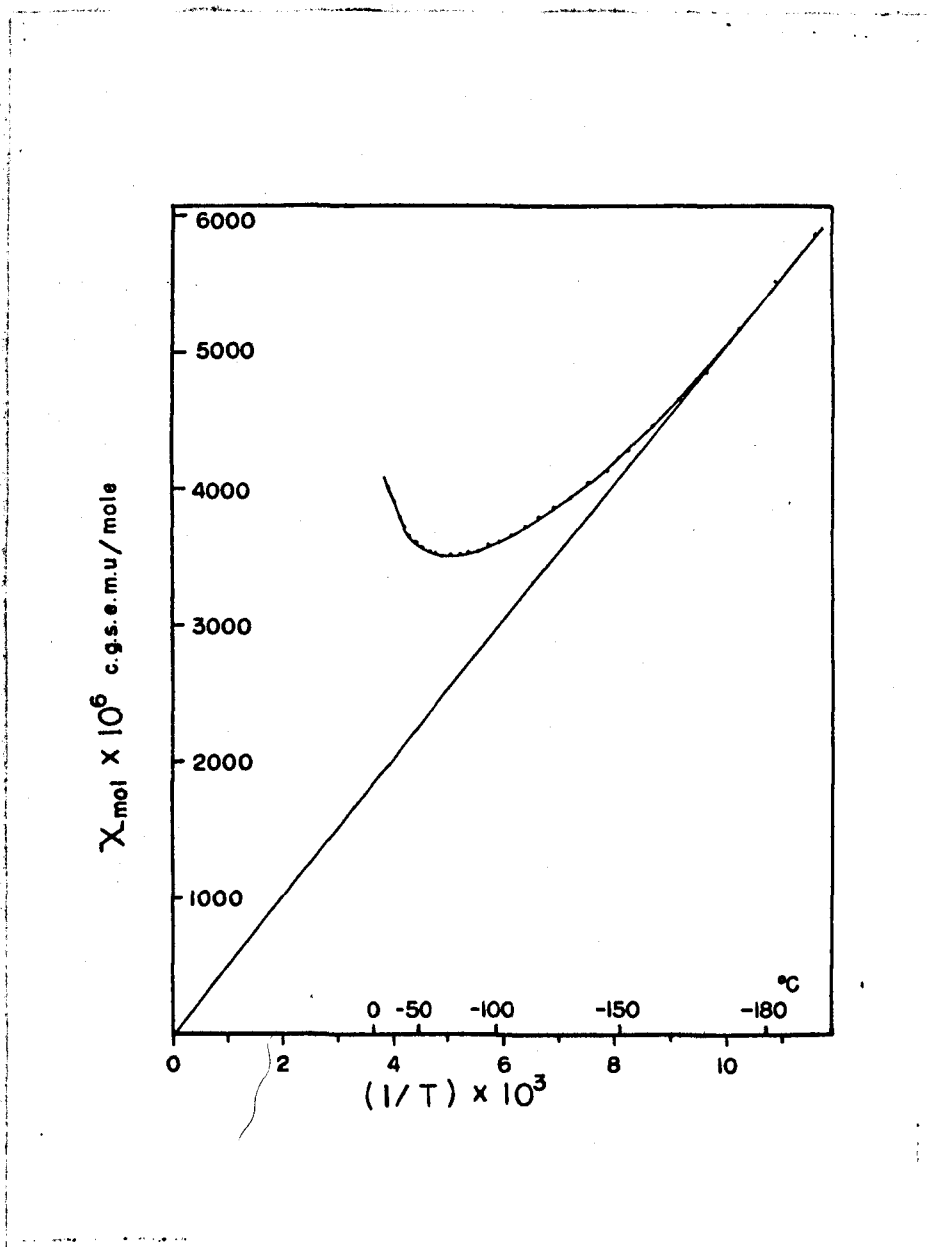


Fig. 7 Abnormal temperature dependency of the molar paramagnetic susceptibility of native ferricytochrome c (pH 3.0).

Table I Extinction coefficients of 22-acetyl-cytochrome c  
at various pH values

pH	$\epsilon_{\lambda}$							
	Oxidized form				Reduced form			
	620	528	490	Soret	550	534	521	Soret
7.4	2.8	8.0	5.3	117.3	23.9	7.6	14.6	137.5
6.0	3.1	7.5	6.7	130.0	24.0	7.6	14.7	137.0
5.1	3.4	6.8	6.8	135.0	23.8	7.6	14.7	135.0
4.5	3.6	6.8	7.0	139.5	24.4	7.8	14.9	137.0
4.0	3.9	6.1	7.1	142.2	23.8	8.3	15.5	135.0

These values were calculated based on the absorption spectrum of pyridine hemochrome assuming the extinction coefficient at 550 m $\mu$  of the hemochrome to be 29.0  $\text{mM}^{-1} \cdot \text{cm}^{-1}$ .

Table II Effect of pH on autoxidation of 22-acetyl-  
cytochrome c

pH	First order rate constants: $k$ ( $\text{sec}^{-1}$ )	
	Fast reaction	Slow reaction
7.4	-	$0.2 \times 10^{-3}$
6.1	$1.4 \times 10^{-3}$	$0.3 \times 10^{-3}$
5.0	$4.8 \times 10^{-3}$	$0.9 \times 10^{-3}$
4.5	$8.7 \times 10^{-3}$	$2.3 \times 10^{-3}$
4.0	$11.5 \times 10^{-3}$	-

These values were calculated from the results shown in Fig. 2.

Table III Effect of pH on CO-binding ability  
of 22-acetyl-ferrocytochrome c

pH	% of CO-complex
7.4	40
6.1	66
5.5	76
5.0	89
4.5	100
4.0	100

These values were calculated from the results shown in Fig. 4.

Table IV Acetylation of lysine and tyrosine residues  
in cytochrome c

Reaction pH	Ratio of acetic anhydride to $\epsilon$ -amino group	Free $\epsilon$ -amino group	O-Acetyl- tyrosine residue
7.0	4	0.6	0.9
	8	0.5	2.3
	10	0.1	2.4
8.0	2	1.7	0.2
	4	0.5	1.3
	6	0.3	2.1
	8	0.3	2.8

The acetylation of cytochrome c with acetic anhydride were performed under the same conditions as used to prepare 22-acetyl-cytochrome c. The amounts of free  $\epsilon$ -amino groups and O-acetyl-tyrosine residues were measured by the methods of Habeeb and Simpson et al., respectively.