

## Properties and Structure of the Compounds Formed between Cytochrome c and Nitric Oxide \*

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The compound formed between ferricytochrome c and nitric oxide has been found to be diamagnetic. It is proposed to be a ferrocyclochrome c nitrosyl compound from which the ferrocyclochrome c nitric oxide compound can be obtained by alkalization. Other haemoproteins behave similarly. The relationship between the optical absorption spectra of the nitrosyl, nitric oxide and carbon monoxide compounds of some ferrohaemoproteins is discussed. The haemlinked group of cytochrome c that is replaced by nitric oxide cannot be detected as a normal histidine imidazole by acid base titration. The possibility of a sterically hindered imidazole group has to be considered.

It is well known that nitric oxide, NO, has the power of combining with iron-porphyrin compounds containing either ferrous or ferric iron<sup>1-3</sup>. Reactions take place with iron-porphyrin bound to proteins<sup>1,2</sup> as well as in haemi- or haemochromogens<sup>2</sup> or with free haem or haematin under certain conditions<sup>3</sup>. While ferricytochrome c combines easily with NO, the reduced form, ferrocyclochrome c, seems to be very inert to NO, and attempts to produce a distinct compound have so far failed.

The ferrous NO compounds which are all stable exhibit typical haemochrome spectra not very different from those of the corresponding CO compounds. Magnetic measurements on the NO derivative of haemoglobin have shown that this complex holds only one unpaired electron<sup>4</sup> which means that the ferrous iron here is surrounded by six octahedral  $d^2sp^3$  bonds and has all its d-electrons paired. The odd electron detected, originates from the NO molecule and is supposed still to belong to the same atomic grouping in the complex. A similar state of affairs is very probably valid for the other ferrous NO compounds.

The ferric NO compounds also show two-banded absorption spectra in the visible but the bands are more distinct and shifted 5 to 10  $m\mu$  towards shorter wavelengths, as compared with the corresponding reduced form. Most of the ferric compounds are unstable, e.g. ferri HbNO<sup>1</sup> and ferri MbNO which are

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gradually autoreduced. In contradistinction to these compounds ferricytochrome-c-NO is stable for a long period of time.

A discussion of the properties of these ferric NO-compounds in terms of the bonds involved in the iron porphyrin complex has not yet been possible, mainly because of lack of magnetic data for the compounds considered. In the present paper it will be shown that these compounds are diamagnetic and contain ferrous iron. The formation of ferrocytochrome-c-NO as a spectroscopically well characterized compound will also be demonstrated.

## EXPERIMENTAL

### Materials

*Nitric oxide* was generated from sodium nitrite mixed with ferrous sulphate by addition of dilute sulphuric acid. The gas was passed through two traps filled with dilute potassium hydroxide and collected in a glass gasometer over dilute hydroxide with phenolphthaleine added as an indicator.

Commercial *nitrogen* from a gas tank was made oxygen free by passing through two traps with vanadyl sulphate dissolved in dilute sulphuric acid and in contact with amalgated zinc.

*Cytochrome c* was prepared from beef heart according to Paléus and Neilands<sup>5</sup> and contained 0.43 % iron. The haemopeptide was purified after degradation of the cytochrome c by pepsin<sup>6</sup>. These preparations were put at our disposal by Dr. S. Paléus.

*Myoglobin*, prepared from horse muscle was kindly given to us by Mr. Å. Åkeson. We are indebted to Dr. K. G. Paul for a sample of *horseradish-peroxidase*.

### Methods

*The magnetic measurements* were performed in the instrument constructed by Theorell and Ehrenberg<sup>7</sup> and later modified to allow for better temperature control and give better reproducibility in the readings<sup>8</sup>. Since the nitric oxide compound of cytochrome c is more stable at lower temperatures all the measurements were made at +7°C, the lowest temperature at which it was feasible to operate the instrument.

A weighed amount (about 30 mg) of lyophilized cytochrome c was placed in a small Thunberg tube. Since part of the cytochrome was present in the reduced form the sample was first dissolved in 0.25 ml 0.2 N HCl and left in this solution until a spectroscopic test revealed that all the reduced cytochrome c had been autoxidized. The sample was thereafter adjusted to the desired pH and concentration, either by 6 N NaOH and M/15 phosphate buffer to a final volume of 1 ml and pH 7.2, or by water to a final volume of 0.5 ml containing 0.1 N HCl, which together with the protein, gave a pH of about 3. In every case two more Thunberg tubes were filled in an identical way, except that the cytochrome c was omitted. These solutions served as blanks in the magnetic determinations, one being measured before and the other after the sample. The magnetic susceptibility was determined prior to and after treatment with NO. This treatment and the measurement on the NO-derivative were made as follows.

The three Thunberg tubes were placed in an ice bath in order to minimize evaporation when the tubes were evacuated. They were connected to a common three way outlet through which they could be quickly evacuated and subsequently filled with nitrogen. This procedure was repeated seven times. The connection leading to the NO-gasometer was also flushed with nitrogen. The Thunberg tubes were then filled twice with NO and left after the last filling for at least 10 min in order to assure a complete reaction, a condition that had been spectrophotometrically controlled for similar samples. The tubes were again quickly evacuated and thereafter placed in a jacket connected to the magnet thermostat and allowed to equilibrate for 20 min to the desired temperature of 7°C. Immediately before the measurement nitrogen was let in and care was also taken to flush with nitrogen the magnet tube and the glass pipet used for transferring the liquid.

The results were corrected for the diamagnetism of the protein by means of data obtained by measuring in the magnet the reduced form of similar samples of cytochrome c.

*The acid-base titrations* were made on 3 ml samples in a small glass beaker with ground neck and a tightly fitting perspex stopper. Holes were drilled in the stopper for one glass and one calomel electrode, two micrometer burets (Agla syringes), and two tubes for inlet and outlet of the gas. A rubber collar was placed in each hole and sealed to the perspex and the inserted glass tube by paraffin. To ensure complete tightness the perspex stopper was provided with a collar and covered with a one cm thick layer of mercury. As an extra precaution the outlet tube was immersed in a vessel filled with mercury to give a constant overpressure in the vessel. Before entering this the gas was freed from traces of acid by passing through a trap filled with dilute potassium hydroxide. Temperature was kept constant by streaming thermostated water in an outer water jacket and stirring was achieved by a magnetic stirrer. 15 to 30 mg cytochrome c was dissolved in 3 ml 0.2 M sodium chloride. 50  $\mu$ l of this solution was taken for spectrophotometric determination of the concentration after dilution and reduction. The sample was brought to a pH below 3 by addition of 0.4 N HCl and left there in contact with air and with slow stirring for about 1 h to ensure complete autoxidation. A stream of nitrogen was started and the ferricytochrome c was titrated with 0.4 N NaOH up to pH 10 and then titrated back with 0.4 N HCl to a pH just below 5. Nitric oxide was now introduced until the colour of the sample had changed. Most of the nitric oxide was replaced by nitrogen and the titration of the NO-compound was made with just enough nitrogen streaming to maintain the small overpressure.

For the amounts of cytochrome c used in the experiments 2 to 4  $\mu$ l of the 0.4 N acid or base corresponded to 1 equivalent. The volumes could be read to every 0.2  $\mu$ l. The pH was measured by a Radiometer pH-meter, model PHM3. The glass electrode was standardised with phthalate and borate buffers before and after each run. In every case the change was less than 0.01 pH unit.

*The spectrophotometric* absorption curves were made with a Beckman DK 2 recording spectrophotometer. Cuvettes with a side bulb, ground stoppers and stopcocks were used. They could be evacuated and washed with nitrogen or argon before NO was let in and later on reaction with the contents in the side bulb could be started. When desired the excess of NO could be removed by evacuation and flushing with argon. All spectrophotometric analyses were made with a Beckman DU spectrophotometer.

## RESULTS AND DISCUSSION

The magnetic susceptibility of the NO-derivative was found to be  $490 \times 10^{-6}$  emu at pH 7.2 and  $220 \times 10^{-6}$  emu at pH 3. In both cases the error of the determination was estimated to be  $\pm 100 \times 10^{-6}$  emu. These data prove that the complex is diamagnetic. The small positive susceptibility values obtained can be explained by some dissociation of the NO-compound, a process that gives two paramagnetic molecules out of one diamagnetic. The experimental value at pH 3 is lower than that at pH 7.2 in spite of the increased susceptibility of cytochrome c at the lower pH<sup>9</sup>. This indicates that the NO-compound is stabilized in the more acid solution. Since the compound is thus found to be diamagnetic as compared with another diamagnetic derivative, ferrocytochrome c, and we are here interested in the "spin susceptibility" there was no reason to correct our data for the "orbital susceptibility" estimated by Griffith for this type of complex<sup>10</sup>.

The diamagnetism of the compound immediately leads to the conclusion that there must be an odd number of NO molecules that combine with the ferric iron, so that the total number of electrons becomes even and the spins can pair out. Earlier equilibrium data support the view that there is only one NO molecule reacting with each cytochrome c molecule<sup>2</sup>. If NO in this

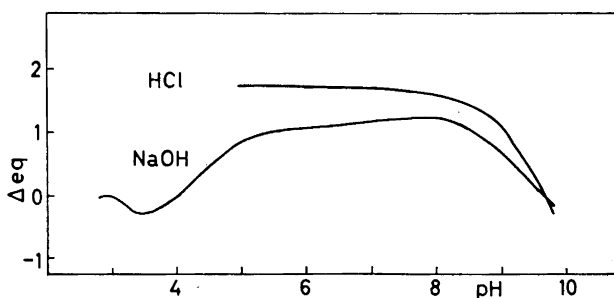
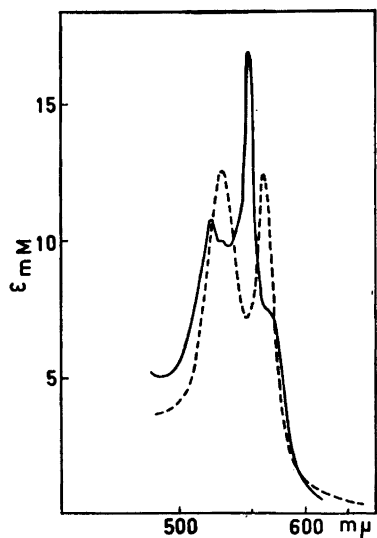


Fig. 1. Difference titration curve of ferricytochrome c after and before reaction with NO.

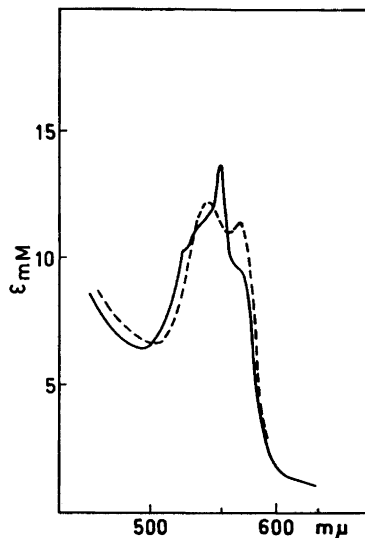
reaction replaces one of the nonporphyrin ligands and this ligand is a histidine residue it might be possible to identify it by a difference titration. When NO reacts the pH of the solution would rise if the  $pK$  of the replaced ligand is equal or higher than the pH of the solution. The pH would be unaffected if the  $pK$  of the ligand is low enough. In all the experiments a drop in pH was instead observed upon reaction with NO and in the different experiments a number of equivalents that varied from slightly less than one to more than four were titrated in the range pH 3 to 5 as seen from the difference curve between the titrations of NO-cytochrome c and ferricytochrome c. A typical curve is given in Fig. 1. Apparently the removal of oxygen had not been complete and some nitrous acid with  $pK = 3.4$  had been formed. 4 to 8  $\mu$ l of oxygen of normal temperature and pressure corresponds to 1 equivalent of cytochrome c in the present experiment. The amount of oxygen dissolved in the standard acid and base used can be neglected, since the solutions were stored under vacuum for some time before the burets were filled. The difference curve also reveals that some groups that are titratable above pH 8 in ferricytochrome c have disappeared in the NO-compound. It is quite reasonable that some of the free amino groups in the protein could react with the nitrous acid and thus be transformed into alcoholic hydroxyls.

In no case more than fractions of one equivalent were titrated in the range between pH 5 and 8. All the equivalents titrated below pH 5 are not necessarily artefacts due to nitrous acid and our experiments are compatible with a  $pK$  somewhere between 3 and 5 for the released haemlinked group. This low  $pK$  might either correspond to a carboxyl group or a histidine residue with its  $pK$  shifted from the normal range. Such a shift might be caused by the histidine being embedded between the protein helices as suggested in the proposed structure of cytochrome c<sup>11</sup>. Also surrounding positively charged groups will operate in the same direction by decreasing the local concentration of the hydrogen ions. The possibility that no haemlinked group is replaced and that the NO is capable of joining to the iron through a new seventh bond of considerable strength must be considered highly improbable, *e.g.* because of the symmetry properties of the bonding orbitals of the porphyrin iron.

When NO reacts with ferricytochrome c to form a diamagnetic compound iron might either donate one electron to NO or accept one electron that is given off by the ligand. In the former case the iron would be oxidized to the

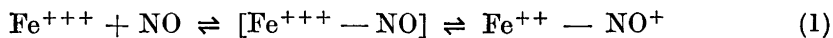


*Fig. 2.* Broken curve: Spectrum of ferrocyanochrome c nitrosyl compound formed in acid or neutral solution from ferricytochrome and nitric oxide. Full drawn curve: Spectrum of the same solution after removal of excess nitric oxide and addition of alkali to obtain pH 12.



*Fig. 3.* Full drawn curve: Transient spectrum 2 min after alkalization of the ferrocyanochrome c nitrosyl compound with excess of NO present. Broken curve: Final spectrum 30 min later.

ferryl state and NO reduced to a negative ion that would be isoelectronic with molecular oxygen. A compound of this type is, however, unlikely to be diamagnetic since the third 3d orbital of the iron would be left empty. The theory predicts electronic configurations of ferryl iron with four unpaired electrons in the high spin state and two, but not zero, unpaired electrons in the low spin state<sup>12</sup>. The secondary peroxide complexes of catalase or horseradish peroxidase and the peroxide compounds of myoglobin are thought to be of this ferryl type<sup>13</sup>. They might be considered to be compounds formed between ferric iron and a free radical representing the rest of the peroxide and have indeed two odd electrons<sup>14,15</sup>. In the second case the iron is reduced to the ferrous state and NO is oxidized to the positive nitrosyl ion, NO<sup>+</sup>, that is isoelectronic with carbon monoxide and by analogy is likely to form a stable compound with the reduced haem iron. The compound formed in the reaction between ferricytochrome c and NO would thus be a ferrocyanochrome c nitrosyl compound as schematically shown in reaction (1).



I

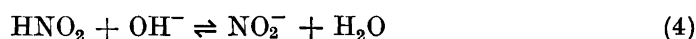
The intermediary complex put within brackets has not been demonstrated and it must be very shortlived if it exists. The spectrophotometric experiments to

be reported below have supplied considerable support for the correctness of the proposed formation of the ferro-nitrosyl compound (I) to the right in formula (1).

When ferricytochrome c reacts with NO at a neutral or acid pH, compound I is rapidly formed. The spectrum, part of which is shown in Fig. 2, has absorption maxima at 417, 530, and 564  $m\mu$ . The dissociation constant for the back reaction is so very small that practically all the excess NO gas can be removed and the compound is still stable at these low pH values. If the formulation of I as a ferro-nitrosyl compound is correct it would, however, be possible for this compound also to dissociate into ferrocytochrome c and the nitrosyl ion:

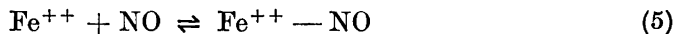


The nitrosyl ions are in equilibrium with the hydroxyl ions, nitrous acid and nitrite ions:



A high pH would thus force reaction (2) to the right. Indeed, if the excess NO gas was removed from an acid solution of I, and this solution was brought under anaerobic conditions to pH 12, a new and stable spectrum quickly appeared. As can be seen from Fig. 2 this spectrum is complicated but the main bands in the visible are situated at 520 and 550  $m\mu$  thus confirming the formation of reduced cytochrome c in a high yield. Less intense bands are situated at 530, 540 and 567  $m\mu$ .

If the alkalization of I is made in the presence of excess NO-gas the complicated spectrum just described is first rapidly formed. However, it gradually changes into a simpler spectrum with absorption bands in the visible at 540 and 567  $m\mu$ , as shown in Fig. 3, and the Soret band at 411  $m\mu$ . Very similar intermediary spectra are observed and an identical end product is formed when oxidized or reduced cytochrome c or a mixture of the two is reacted with NO at pH 12. The compound formed in all these reactions has to be formulated as a complex with nitric oxide bound to the iron of ferrocytochrome c, and is here labeled II:



## II

Compound II has also been shown to be stable at lower pH-values. It was first formed from ferrocytochrome c and NO at pH 12 and then in the presence of NO brought to pH 7.5 by addition of a measured amount of primary phosphate. No marked change in the spectrum was observed after 30 min. At still lower pH-values and in the presence of nitrite compound I is formed by reversal of reactions (2)—(5). Enough nitrite for this reaction sequence is easily formed by incomplete anaerobiosis during exposure to NO, especially if compound II has originally been obtained from I, in which reaction one equivalent of nitrite is formed.

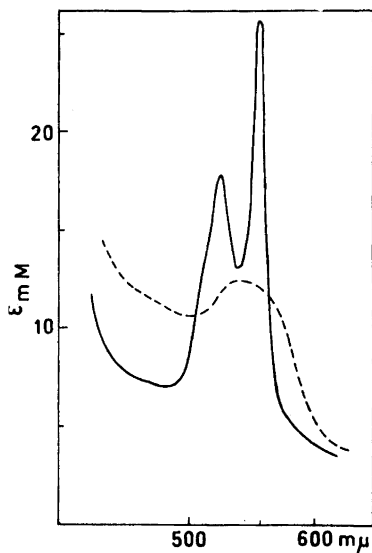


Fig. 4. Spectrum of ferrohaemopeptide at pH 7, full drawn curve, and the products after reaction with NO, broken curve.

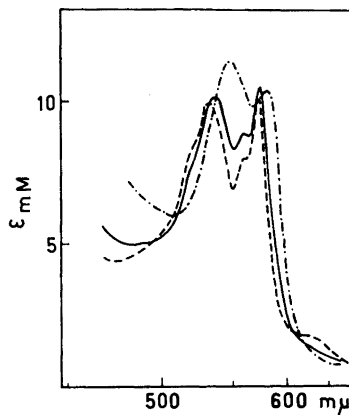


Fig. 5. Transient spectrum of ferrimyoglobin immediately after reaction with NO at pH 7 (---) and the stable spectrum of the ferrinitrosyl compound measured 3 hrs later (—). At pH 10 the later spectrum is immediately formed, and with excess NO the stable spectrum of the ferromyoglobin NO-compound could be measured after 2 hrs (- . - .).

From formula (3) it can be seen that the nitrosyl ion concentration would be expected to increase with the concentration of nitric acid and the concentration of hydrogen ions. This dependence is illustrated by the spectral changes of reduced cytochrome *c* in 10 mM nitrite solutions of different pH. At pH 2 and 4.2 the reduced cytochrome *c* was immediately transformed into compound I. At pH 5.3 the transformation is slower and at pH 7 or higher no reaction at all takes place.

At pH-values below 2 oxidized cytochrome *c* is also quantitatively transformed into I by the action of nitrous acid. This shows that NO must be formed from nitrous acid under these conditions, probably by disproportionation into nitric acid and nitric oxide. At pH 5.3 the spectrum of oxidized cytochrome *c* is not affected by added nitrite.

The inability of ferrocytochrome *c* to react with NO in neutral solution parallels its behaviour towards CO and O<sub>2</sub>. These molecules are all small and uncharged but can nevertheless not reach and react with the iron. A very close and tight structure is thus necessary around the haem of cytochrome *c*. This structure is opened slightly when the cytochrome is oxidized, and the NO molecule can now penetrate and react with the iron. Molecules of similar size but with an electrical charge, *e.g.* CN<sup>-</sup>, can still not reach the iron because

Table 1. Comparison of the absorption band positions of some ferrohaemoprotein derivatives. Data on the CO compounds of cytochrome c and catalase are taken from Refs.<sup>17,18</sup>, respectively.

Haemoprotein	Derivative	Wavelength of absorption maximum in $m\mu$		
Cytochrome c	NO <sup>+</sup>	563	530	417
	CO	563	530	—
	NO	567	540	411
Myoglobin	NO <sup>+</sup>	575	537	420
	CO	578	542	423
	NO	580	548	421
Haemoglobin	NO <sup>+</sup>	568	532	—
	CO	568	538	419
	NO	576	542	—
Horseradishperoxidase	NO <sup>+</sup>	570	539.5	423
	CO	573	542	423
	NO	575	545	423.5
Catalase	NO <sup>+</sup>	576	540	—
	CO	580	545	—
	NO	—	—	—

of a potential barrier due to charge groups in the protein moiety. The same potential barrier helps to stabilize the ferro-nitrosyl complex formed, by protecting the positively charged nitrosyl ion from "leaking" out. This protection is strongest in acid solutions, when positive charges dominate on the protein surface, and will decrease with increasing pH, thus explaining the observed pH dependence of the stability of the ferrocycytochrome c nitrosyl complex. The compact structure itself will also be opened at higher pH-values. Ferrocycytochrome c can now combine with NO or CO or it can be oxidized by O<sub>2</sub>. The stability of the ferro-NO compound when neutralised also demonstrates that an uncharged molecule can be captured inside the closed structure surrounding the haem.

The spectra and reactions of some compounds related to cytochrome c have also been explored. Thus the ferrihaemopeptide gives with NO a compound with  $\alpha$ - and  $\beta$ -bands that are indistinguishable from those of I. This is to be expected, since there is the same configuration around the iron whether NO reacts with the ferrihaemopeptide from the opposite side to the single histidine imidazole present, or with ferricytochrome c by replacement of one of the coordinated histidines. As distinct from ferrocycytochrome c the ferrihaemopeptide that has no closed structure reacts with NO in neutral solution as is seen in Fig. 4. The spectrum is poorly defined and probably represents a mixture of compounds. Possibly more than one NO molecule could be coordinated.

When NO reacts with ferrimyoglobin at pH 7 a distinct but rather complicated spectrum is immediately formed, see Fig. 5. This spectrum is not stable but in a couple of hours changes into one where the main absorption maxima are at slightly longer wavelengths. The concomitant disappearance of a weak



absorption at 630  $m\mu$  indicates that some ferrimyoglobin is slowly transformed into the ferronitrosyl compound, which itself is stable for many hours in the neutral solution. At pH 10 the ferro-nitrosyl compound is formed immediately. but in the presence of excess NO it is completely transformed into the ferromyoglobin NO-compound in less than two hours. The latter can be formed directly from ferromyoglobin and NO at pH 10 and also at pH 7. All the main features of these reactions of myoglobin are compatible with a structure where the haem is more exposed than in cytochrome c.

It is very interesting to compare the absorption maxima of the compounds listed in Table 1. Changing the ligand to the reduced haem iron from  $\text{NO}^+$  to CO and then to NO the general trend is that the wavelengths of the  $\alpha$ - and  $\beta$ -bands increase slightly, while the  $\gamma$ -band remains constant or is somewhat shifted towards shorter wavelengths. Both  $\text{NO}^+$  and CO have empty orbitals and are capable of forming a weak double bond with the iron, thus pulling electrons away from the iron. The effect of the positively charged  $\text{NO}^+$  will be more pronounced than for the neutral CO. NO is not capable of forming a double bond, because there is no empty orbital, and its effect will be even smaller. The pull on the electrons will be transmitted from the iron out through the conjugated bond system of the porphyrin. The excitations corresponding to the  $\alpha$ - and  $\beta$ -bands are believed to be associated with a charge-redistribution resulting in a shift of the electrons towards the periphery of the porphyrin disc<sup>16</sup>. The pull on the electrons exerted by a ligand at the iron will make these excitations more difficult and hence will shift the bands towards shorter wavelengths, as observed. The  $\gamma$ -excitation is associated with less pronounced charge-redistribution<sup>16</sup>, the direction of which cannot be settled with certainty from the data of the  $\gamma$ -bands in Table 1.

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