BBA 3941

ON THE ANOMALOUS INTERACTIONS OF LIGANDS WITH RHODOSPIRILLUM HAEM PROTEIN

S. TANIGUCHI* AND M. D. KAMEN

Department of Chemistry, University of California, San Diego, La Jolla, Calif. (U.S.A.)

(Received September 5th, 1962)

SUMMARY

The spectroscopic responses of *Rhodospirillum rubrum* haem protein to a variety of ligands over the pH range 5.2–12 have been examined. Only small uncharged molecules, *e.g.*, NO and CO, are able to penetrate to the prosthetic haem groups of the protein. NO reacts not as a ligand, however, but causes changes which appear to be the result of oxidative alterations either of the porphyrin moieties or of reactive groups in the immediate peptide environment, or of both. CO, on the other hand, reacts as a true ligand. However, the affinity of rhodospirillum haem protein for CO is much less than that of most other haem proteins; the half-saturation value for ferro-rhodospirillum haem protein–CO formation is about 0.015 atm at all pH values tested. The atypical variation in photodissociability of ferro-rhodospirillum haem protein–CO with pH, noted previously, is confirmed.

INTRODUCTION

Despite its spectroscopic similarity to myoglobins, the variant haem protein (RHP) of the purple photosynthetic bacteria[1] binds none of the usual ligands at neutral pH except CO, with which it reacts when in its reduced form[1,2]. This fact, together with certain chemical and structural similarities to cytochromes of the "*c*" class, has prompted the suggestion that it be considered a prototype for a new class of haem proteins, to be called "cytochromoids"[3].

No systematic study of the response of RHP to ligand reagents when subjected to a variety of conditions has been made. The few observations reported have been restricted to interactions of the native form of RHP at neutral pH with a relatively small number of ligands. It has been established, also, that RHP undergoes progressive modification, when it is incubated in media of increasing alkalinity, particularly in its oxidized form[4]. These changes, manifested both spectrophotometrically[4] and immunochemically[5], are expected to be associated with increased tendency for ligand binding.

Abbreviation: RHP, rhodospirillum haem protein. The nomenclature for various types of haematin compounds follows suggestions of the Commission on Enzymes, International Union of Biochemistry[3].

* International Fellow, Rockefeller Foundation, 1961, on leave from Nagoya University, Nagoya (Japan).

We now report results of experiments performed to extend previous spectroscopic observations on the behavior of native RHP at neutral pH, as well as to test for the anticipated changes in reactivity. We have studied the spectroscopic responses of RHP over a range of pH from 5.2 to 12.0 when exposed to potassium cyanide, sodium azide, sodium fluoride, hydrazine sulfate, hydroxylamine sulfate, sodium nitrite, nitrosobenzene, nitrous oxide, and nitric oxide—typical ligands for the haem proteins of the "open" type, such as the haemoglobins and myoglobins, in which the haem group is accessible.

In addition, we have repeated and extended earlier observations on the interaction between RHP and CO, which provide a basis for comparison of CO and NO as reactants. We find that CO is the only compound of those tested which reacts solely as a ligand.

MATERIALS AND METHODS

Preparation of RHP

RHP was prepared in pure crystalline form in the cold (4°) by a modification of the method described previously by HORIO AND KAMEN[4]. Lyophilized powders of *R. rubrum* cells, grown for 4 days in the light in the medium described by ORMEROD *et al.*[6], were mixed in a Waring blendor with 0.1 M sodium acetate buffer (pH 5.2) in a ratio 1:7 (w/v). The mixture was stirred in the cold for approx. 12 h and then centrifuged at 10000 × *g* for 30 min. The clear supernatant fluid was passed through a column packed with Amberlite CG-50 Type II (mesh 100–200) equilibrated with 0.1 M sodium acetate buffer (pH 5.2). The proteins adsorbed on the top of the column were washed thoroughly by repeated passage of the same buffer through the column to remove undesired soluble contaminant proteins and small particles. The resin containing the colored adsorbate was removed from the column, suspended and rinsed with cold distilled water a few times. The washed resin was packed in a column and all haem proteins adsorbed were eluted with 2.0 M ammonium phosphate buffer (pH 7.0). The collected eluate, containing both RHP and cytochrome c_2, was brought to approx. 50% saturation by addition of finely powdered ammonium sulfate. To the supernatant fluid obtained by centrifugation after standing for 1 h, ammonium sulfate powder was added to a concentration of approx. 70% saturation. Almost all the RHP was collected in the precipitate obtained by centrifugation after standing for 1 h, leaving the major fraction of cytochrome c_2 in the supernatant fluid. The precipitate was dissolved with a minimum of cold distilled water and approx. 5 mg of sodium dithionite added to this solution to convert all remaining cytochrome c_2 to its reduced form. Unlike the reduced cytochrome, RHP after reduction reverted rapidly to its original oxidized form, owing to its autoxidizability. This treatment was employed to facilitate separation in the following chromatographic procedure by taking advantage of the fact that the reduced form of the cytochrome could be eluted rapidly under conditions in which RHP was immobile. The RHP solution was dialyzed first against more than 10 vol. of distilled water and next against 0.1 M acetate buffer (pH 5.65) with four changes of buffer. The dialyzed solution was charged on a column packed with the same resin equilibrated with the same acetate buffer. Under these conditions, the reduced form of the cytochrome moved down the column while the brown band of RHP diffused slightly, but remained near the top of the column. The column was washed thoroughly by repeated percolation with

the same buffer. After the complete removal of cytochrome c_2, RHP was eluted with 2.0 M ammonium phosphate buffer (pH 7.0). Further treatment to produce pure crystalline RHP followed the ammonium sulfate fractionation procedures described previously[4]. The ratio of absorbancy of the oxidized form at 638 mμ to that at 277 mμ was 0.130–0.132, in agreement with the ratio obtained by HORIO AND KAMEN[4] for their purest samples. The suspension of RHP crystals in the ammonium sulfate solution (pH 7.0) was stored in the cold. Prior to use, the RHP was freed of the ammonium salt by serial dialysis for 15 h against 0.001 M NaCl solution.

Buffers and ligands

Experiments were made at four values of pH—5.2, 7.0, 9.0 and 12.0. These were obtained by use of appropriate potassium phosphate buffers (final concentration, 0.1 M) made up from 0.5 M stock solutions of the mono-, di-, and triphosphates. In order to distinguish the effects of ligands from pH effects on the spectra of RHP, the pH of the mixture of RHP, buffer, and ligands was checked before and after each spectrophotometric measurement. Deviations in pH during the spectrophotometric measurements were well within ± 0.2 pH unit.

All reagents were C.P.-grade chemicals (Fisher Chemical Co.) with the exception of nitrosobenzene, which was obtained from K and K Laboratories, Inc., Jamaica, New York. The nitrosobenzene was freshly prepared as a stock solution ($8 \cdot 10^{-2}$ M) by dissolving the crystals in a minimum amount of ethanol. NO and CO were supplied by the Matheson Co. Spectra were determined with a Cary recording spectrophotometer, type 14. To permit spectral plots on the same scale range of absorbancies, different concentrations of RHP were used in the two spectral regions, *i.e.*, 14–38 mμmoles/ml in the range 450–700 mμ, and 2.0–2.6 mμmoles/ml in the range 300–450 mμ. All measurements were performed at room temperature (23°–25°).

For study of the effect of ligands other than NO and CO, a cuvette (1 cm optical path) received an appropriate quantity of RHP solution, buffer, ligands, and deionized water to make a total volume of 3.5 ml. The reduced form of RHP was obtained by addition of trace quantities (approx. 2 mg) of solid sodium dithionite. Except for experiments with NO and CO, spectrophotometric recording was started 2 min after the addition of ligands. The maximal concentration for all ligands tested was 0.01 M except for nitrosobenzene, which was 0.005 M.

In experiments with NO and CO, a Thunberg-type cuvette (1 cm optical path) adapted for gas changes was used. For anaerobic measurements with oxidized RHP, after 7-fold flushing of all connections leading to the gasometer or gas tank with high-purity argon (purchased from Liquid Carbonic Co.) the cuvette was evacuated and filled to atmospheric pressure with argon gas. The cuvette then was flushed seven times with argon, after which the same cycle of gassing operations was repeated with NO or CO. Eventually, the cuvette was filled with NO or CO to 1 atm pressure. To facilitate equilibration, the cuvette filled with these gases, especially in the case of NO, was shaken gently for 1 min after each refilling operation. For anaerobic measurement with the reduced form of RHP, about 2 mg of sodium dithionite were placed in the thoroughly dried side arm, and mixed with RHP solution, by tipping under argon prior to introduction of NO. In experiments on saturation of RHP with NO and CO, these gases were diluted in a definite proportion with argon gas in a gasometer (under 1 atm pressure), and flushed into a cuvette in the same manner

as above. For each run it was spectrophotometrically ascertained that during the gassing procedure RHP reached an equilibrium with these gases at each partial pressure applied.

Control experiments with NO-saturated buffer showed considerable absorptions with variable distribution of peak absorbancies in the region of wavelength below 400 mμ. To cancel the absorption arising from NO alone, spectra of the RHP–NO products were always determined against a reference cuvette containing the corresponding mixture, prepared in exactly the same manner except that RHP was replaced by 0.001 M NaCl solution. Absorption peaks for NO-saturated salt solutions under 1 atm NO pressure were especially noticeable at acidic pH. For example, under these conditions at pH 5.2, the highest peak in the region 355–360 mμ attained an absorbancy of 0.2–0.3. Therefore, we abandoned attempts to measure the RHP spectra at wavelengths shorter than 350 mμ, and attached significance only to measurements at longer wavelengths. To assure completion of reaction, the spectra which resulted from the interaction with NO were first monitored 5–10 min after the exposure to NO and a few times more thereafter until no further change occurred in spectra (30 min–1 h later, depending on the pH of the reaction).

In addition to effects of NO, we also examined the effect of N_2O on RHP spectra at those four different pH's. However, no spectral response was detected, even at 1 atm pressure of N_2O. Therefore, the possibility that the effects of NO on RHP described below involved effects of N_2O, which might be produced in minute quantities due to the reaction of NO with dithionite added to reduce RHP, was eliminated.

To observe the photodissociability of RHP–NO or RHP–CO compounds, the effect of illumination for 30 min with white light was observed at intensities of approx. 400–1000 ft candles at 23°. In measuring the spectra of the photodissociable reduced RHP–CO compound the following precautions were taken to minimize photodissociation during measurement, which was considerable especially in the experiments at low CO pressure (0.01–0.001 atm). Thus, before monitoring the spectra, preincubation at 23° was carried out for 30 min in complete darkness. Spectra were monitored immediately after preincubation in the Cary spectrophotometer in the dark with a small slit (approx. 0.05 mm) and with rapid scanning (250 mμ/10 sec). Measurement over the needed wavelength range (450–400 mμ) was completed within 25 sec, including the time to set the cuvette in the photometer chamber and to adjust the pen position, and gave reproducible absorbancies in numerous trials. Because of the short times and low light intensities involved in these manipulations, the extent of photodissociation of RHP–CO compound during measurement could be discounted.

RESULTS

Effects of potassium cyanide, hydroxylamine sulfate, sodium azide, sodium fluoride, hydrazine sulfate, sodium nitrite and nitrosobenzene on RHP spectra

The effects of these ligands were examined at pH 5.2, 7.0, 9.0 and 12.0, except for potassium cyanide and sodium nitrite at pH 5.2 and for hydroxylamine sulfate at pH 12.0, which could not be tested because of their instability at these pH values. In all cases, spectral changes observed were not significant. Small changes of spectra sometimes observed, compared with the control cuvette without ligand, were caused by slight shifts of pH incidental to additions of ligands. The effects of these ligands

were checked after prolonged incubation for 1 h and, for nitrosobenzene, for 3 h; still, no significant responses were detected. It was sometimes observed at pH 12.0 that the addition of cyanide, azide, and fluoride to ferro-RHP produced a slight attenuation of the two ferro-haemochrome peaks at 550 mμ and 521 mμ exhibited by free ferro-RHP at this pH. However, these changes were insignificant compared with the effects caused by NO or CO at pH 12.0 (see Fig. 4).

Reaction of NO with RHP at pH 5.2; comparison with CO-reaction

The most complete reactions occurred with ferri-RHP at this pH. In Fig. 1 we exhibit the spectra of the products formed with ferri- and ferro-RHP. We found it necessary to use lower concentrations of the ferro form to avoid turbidity because of the low solubility of ferro-RHP relative to that of ferri-RHP.

On exposure to NO, ferri-RHP quickly lost absorbancies at its characteristic maxima, 497 mμ and 638 mμ, and simultaneously developed two new absorption bands with maxima at 563 mμ and 530 mμ. This reaction was complete in 10 min; the final spectrum showed a slightly greater absorbancy at 563 mμ than at 530 mμ. Molar absorbancy indices (in l/mol·cm) were $26.0 \cdot 10^3$ and $25.2 \cdot 10^3$ at 563 mμ and

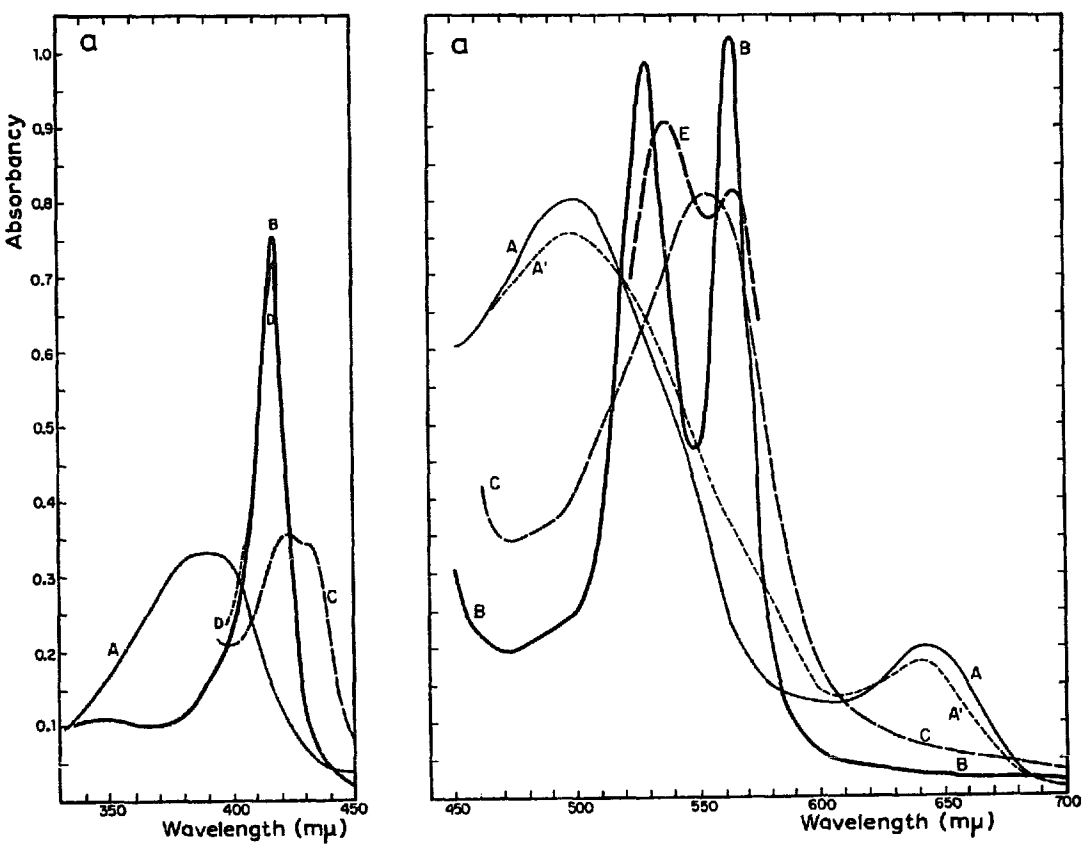

Fig. 1a. Reactions of NO and CO with RHP at pH 5.2. RHP concentrations, approx. 37.8 and 2.0 mμmoles/ml, were used for the measurements in the visible (700–450 mμ) and Soret (< 450 mμ) regions, respectively. Spectrum A, oxidized form before reaction; spectrum B, oxidized form after reaction with NO, 10 min after last gas exchange; spectrum A', starting with oxidized form after reaction with NO (spectrum B), NO was evacuated and replaced with argon and the pH was adjusted to approx. 7.0. This spectrum was recorded 30 min afterward; spectrum C, reduced form before reaction with CO; spectrum D, reduced form after reaction with CO in the dark, 30 min after the last gas exchange; spectrum E, reduced form after reaction with CO, 30 min dark incubation.

530 mμ, respectively, relative to the previously determined value, $5.86 \cdot 10^3$ for the "haematin" peak[4] at 638 mμ and pH 7.0.

The visible spectrum which resulted when ferro-RHP was exposed to NO showed two new peaks which, while less prominent than those found for the ferri-RHP–NO products, were nevertheless at almost the same wavelengths. However, another lesser peak was observed at approximately 480 mμ (Fig. 1b).

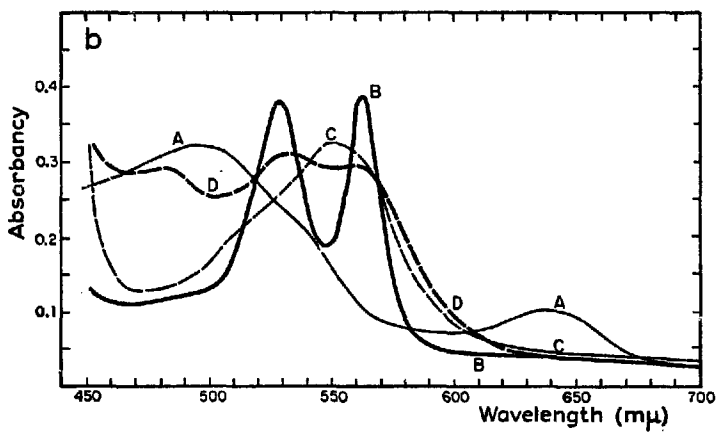

Fig. 1b. Reaction of NO with RHP at pH 5.2. Concentration of RHP approx. 14.0 mμmoles/ml. Spectra A and B as in Fig. 1a; spectrum C, reduced form; spectrum D, reduced form after reaction with NO.

The two ferrohaemochrome-like peaks obtained for either ferri- or ferro-RHP after exposure to NO were compared to those of the ferro-RHP product found after reaction with CO at 1 atm in the dark at the same pH and concentration of protein. As seen in curve E of Fig. 1a, which shows only the main absorption bands, the ferro-RHP–CO product spectrum peaks were easily differentiated from those of either ferri- or ferro-RHP–NO spectra by the placement of the absorption maxima for the CO product at 565 mμ and 536 mμ and by the lack of resolution of the two peaks.

The NO products were remarkably stable, especially those formed from ferri-RHP. We found no change in spectrum even after solutions stood at this pH for 1 week at 4°. Evacuation and replacement of NO with argon produced only a slight change in the spectrum. However, a shift in pH to 7.0, brought about under argon by addition of K_3PO_4, produced the spectra characteristic of free ferri-RHP. The phosphate, present in the side-arm of the Thunberg cuvette, was tipped into the reaction mixture. The quantity needed to bring the pH to 7.0 was determined in prior pilot experiments. The spectrum obtained after 30 min of incubation at pH 7.0, and recorded as curve A′ in Fig. 1a was identical with that for free ferri-RHP at the same concentration.

The spectrum of ferri-RHP-NO products could be obtained by return of the pH to 5.2. This was accomplished by opening the cuvette to air, titrating the reaction mixture with a minimal volume of concentrated HCl to pH 5.2, and then repeating the gassing procedure with final addition of NO. This cycle of pH change was repeated successfully once more. We found that the spectrum of the ferro-RHP-NO product also reverted to that of the free ferri-RHP on exposure to air.

Spectral changes were also found, as expected, in the Soret region. As seen in

Fig. 1a, both ferri- and ferro-RHP gave rise to essentially identical spectra within less than 10 min. The original Soret maxima of free RHP were lost completely and replaced by a single sharp maximum at 417 mμ. The absorbancy of the new Soret peak was approximately double those of the original Soret maxima, with molar absorbancy approx. $370 \cdot 10^3$. As before, the original spectrum of free ferri-RHP was recovered by aeration.

The Soret peak of the ferro-RHP–CO compound (1 atm CO, pH 5.2) appeared at 415 mμ, significantly displaced towards the blue compared to the NO products. The molar absorbancy value of the CO compound was significantly greater, however, being approximately $500 \cdot 10^3$.

Reaction of NO with RHP at pH 7.0

Spectra obtained at pH 7.0 were much like those seen at pH 5.2, but apparently reactions were less complete (Fig. 2), as judged by partial persistence of the haematin peak of free ferri-RHP. Reactions were sufficiently sluggish so that several sets of transient spectra could be seen before appearance of stable spectra (30 min after initial exposure). If we assumed reaction at pH 5.2 to have been complete, based on the total disappearance of the 638-mμ haematin peak, then we could estimate from the measured changes in absorbancies, either at 638 mμ (decrease) or at 563 mμ (increase), that approx. 55 % of the ferri-RHP failed to react. As seen in curve B,

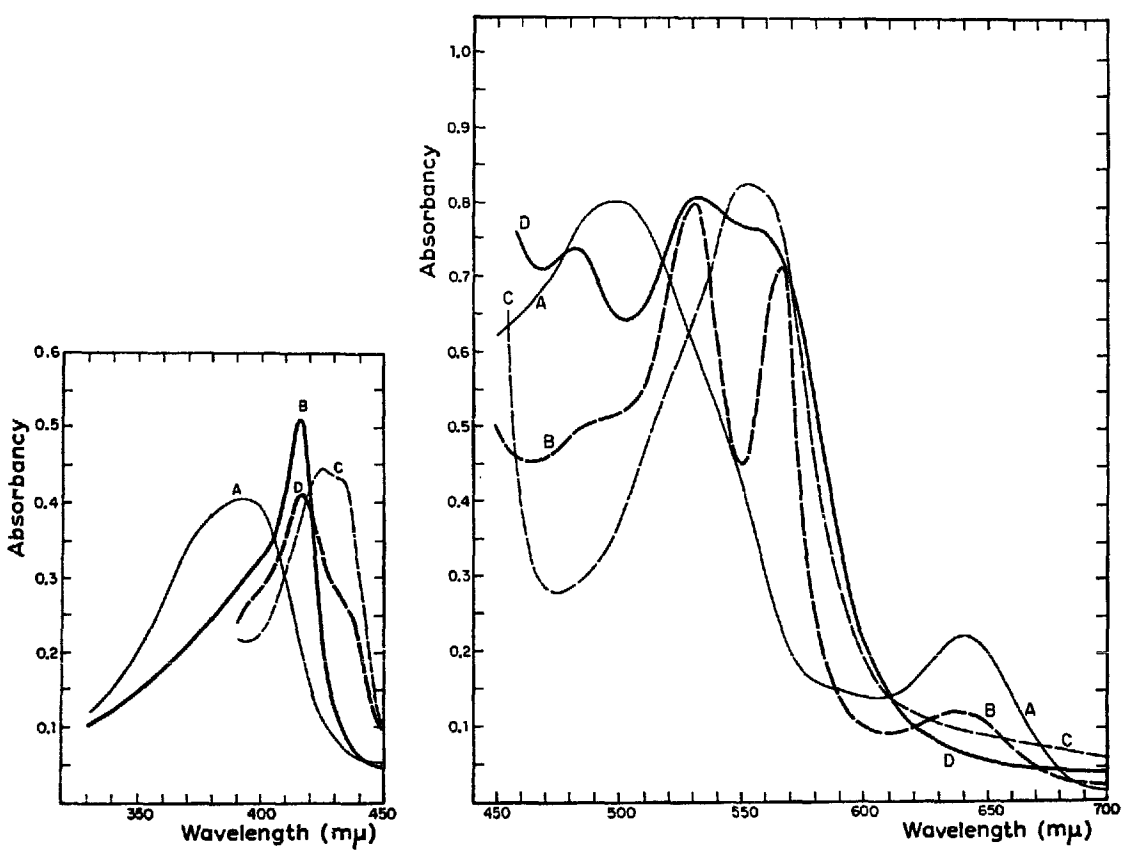

Fig. 2. Reaction of NO with RHP at pH 7.0. RHP concentrations of approx. 37.8 and 2.6 mμmoles per ml were used for the measurements in the visible and Soret regions, respectively. All spectra are those of final stable products. Spectrum A, oxidized form before reaction; spectrum B, oxidized form after reaction, 30 min after last gas exchange; spectrum C, reduced form before reaction; spectrum D, reduced form after reaction, 30 min after the last gas exchange.

Fig. 2, a minor peak in the ferri-RHP-NO spectrum appeared at approx. 482 μm which was not seen at pH 5.2.

Reaction with ferro-RHP was also slow and required at least 30 min before appearance of the final stable product spectrum. Again the two ferrohaemochrome-like peaks at 560 and 530 mμ were in evidence, but not well resolved, as at pH 5.2; in addition, the small peak at approx. 482 mμ emerged where a trough had been in the original spectrum of free ferro-RHP.

Spectral responses in the Soret region also mimicked those found at pH 5.2. The original Soret maximum of free ferri-RHP at 390 mμ shifted after 30 min to a new slightly enhanced peak at approx. 417 mμ. A similar absorption maximum, of somewhat lesser absorbancy, was seen in the reaction of ferro-RHP with NO after a 30-min incubation.

Thus, it could be concluded that reactions similar to those at pH 5.2 occurred less completely and rapidly at pH 7.0. Also, that these were "reversible", as at pH 5.2, by exposure to air, after removal of NO from the gas phase; in any case we recovered ferri-RHP by this sequence of operations whether we started with ferri- or ferro-RHP.

Reaction of NO with RHP at pH 9.0

As at pH 7.0, reactions at pH 9.0 were slow; the spectrum of the final product formed with ferri-RHP was not established until 1 h had passed (Fig. 3). A transient spectrum (curve B, Fig. 3) showed very slight evidence of the existence of the two

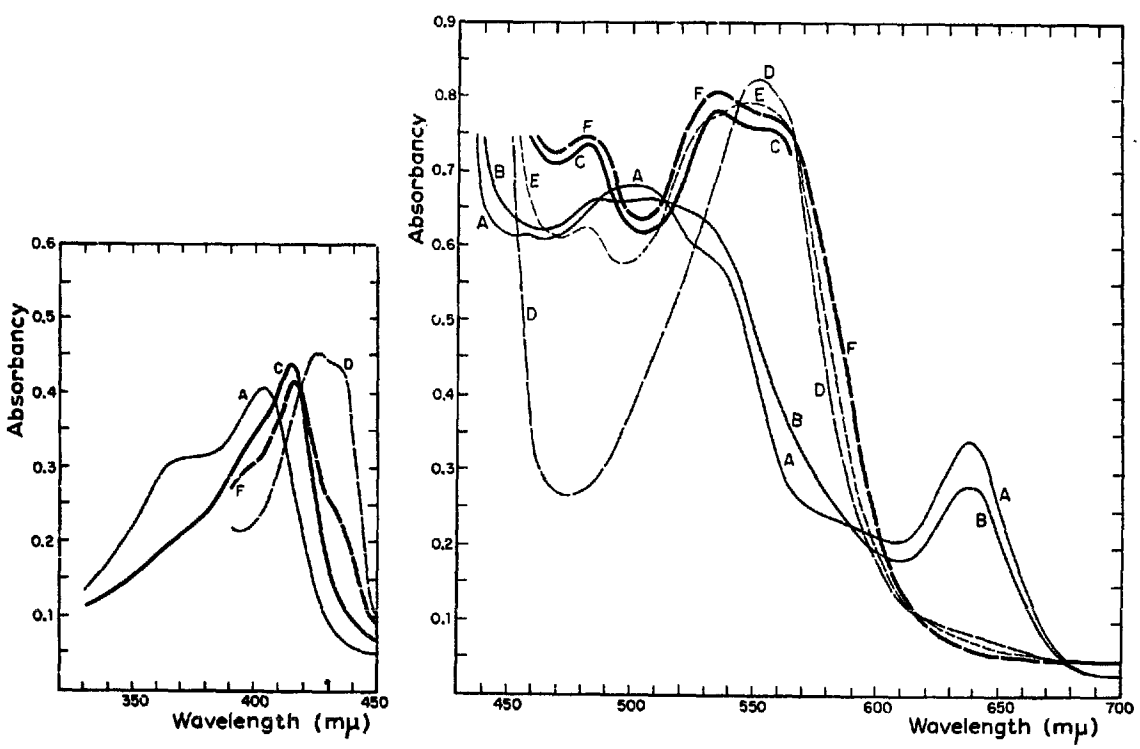

Fig. 3. Reaction of NO with RHP at pH 9.0. Same RHP concentrations as at pH 7.0 were used. Spectrum A, oxidized form before reaction; spectrum B, oxidized form after reaction; transient spectrum recorded 5–10 min after the last gas exchange; spectrum C, same as spectrum B, but recorded 1.0 h later. This is the final stable spectrum; only double-peaked part is shown, however, the whole shape is as in spectrum F; spectrum D, reduced form before reaction; spectrum E, reduced form after reaction; transient spectrum recorded 5–10 min after the last gas exchange; spectrum F, final stable spectrum recorded 30 min later than spectrum E.

ferrohaemochrome-like peaks seen so clearly at pH 5.2. The spectrum of the stable product finally obtained with ferri-RHP was indistinguishable from that found with ferro-RHP; both exhibited complete absence of the 638-mμ peak.

In the Soret region, the broad absorption maximum at 360–370 mμ characteristic of free ferri-RHP at this pH, disappeared to be replaced by a single peak at approx. 417–418 mμ, like that found previously at pH 7.0. Thus, reactions at pH 9.0 produced effects on the Soret bands which resembled those seen at pH 7.0. Spectra obtained for the final products at this pH, as well as under more alkaline conditions (next section), were essentially identical whether the initial reactant was ferri-RHP or ferro-RHP.

Reaction of NO with RHP at pH 12.0

Reactions at this pH, either with ferri- or ferro-RHP, proceeded faster than those at pH 7.0 or 9.0. As seen in Fig. 4, the spectrum of free ferri-RHP at this pH did

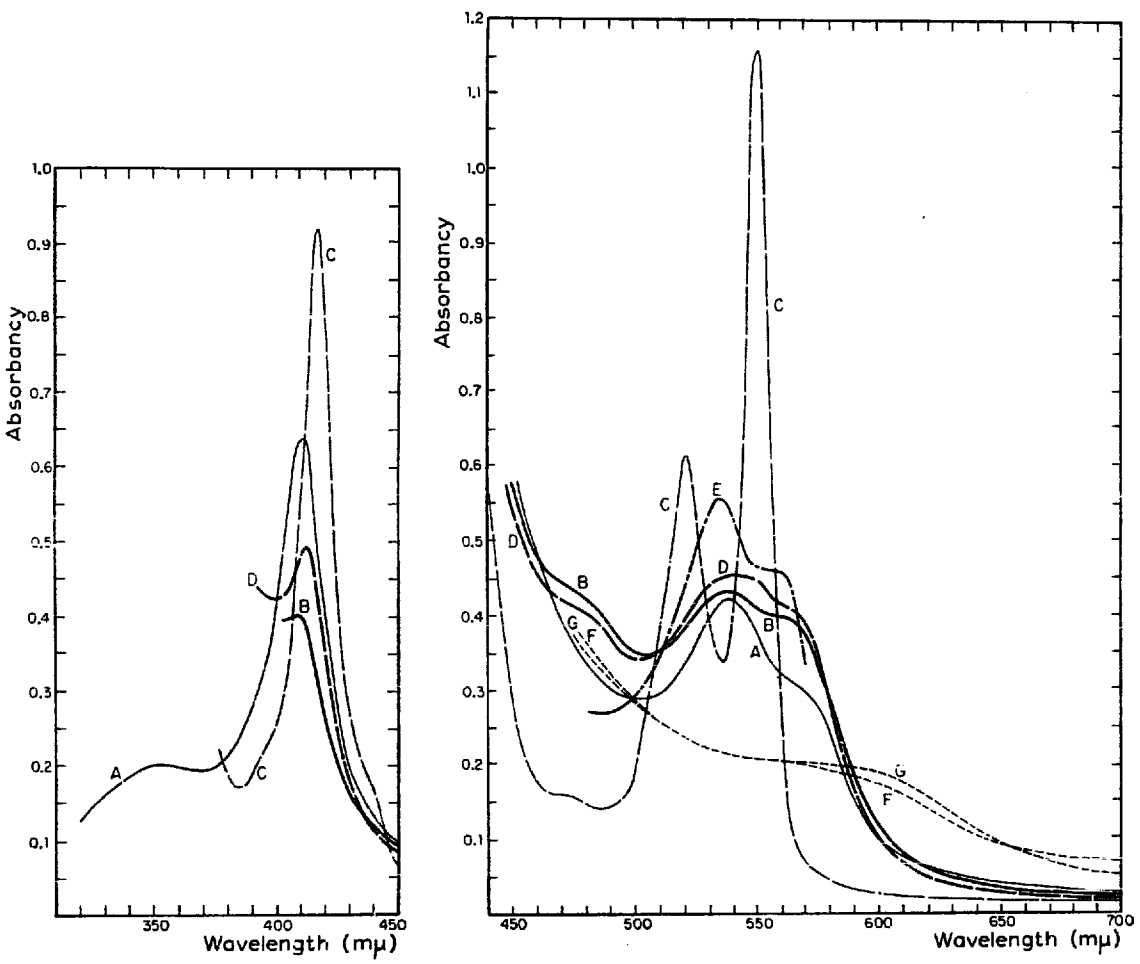

Fig. 4. Reactions of NO and CO with RHP at pH 12.0. RHP concentrations of approx. 21.8 and 2.6 mμmoles/ml were used for the measurements in the visible and Soret regions, respectively. Spectrum A, oxidized form before reaction; spectrum B, oxidized form after reaction recorded 5–10 min after the last gas exchange; spectrum C, reduced form before reaction; spectrum D, reduced form after reaction with NO, 5–10 min after the last gas exchange; spectrum E, reduced form after reaction with CO, 5–10 min after dark incubation; spectrum F, starting with the oxidized form after reaction with NO (spectrum B), NO was removed by evacuation and replaced with argon, then subsequently exposed to air for 10 min; spectrum G, reduced form after reaction with NO (spectrum D) was treated in the same way as in spectrum F.

not show the acid haematin peak characteristic of neutral oxidized RHP, but rather an alkaline ferrihaemochrome spectrum, which was replaced by a blurred ferrihaemochrome-type spectrum with maxima at approx. 540 mμ and 565–570 mμ. Occurrence of the hump around 480–485 mμ was also noted as at pH 7.0 and 9.0. The characteristic ferrohaemochrome-like spectrum of free ferro-RHP at this pH, which consisted of two peaks at 550 and 521 mμ, was attenuated immediately upon exposure to NO, and eventually abolished. The final spectrum, obtained after 15 min, showed the same blurred ferrihaemochromogen-like peaks as those produced starting with ferri-RHP.

CO, at pH 12.0, produced different effects (curve E, Fig. 4). Thus, the peaks of the final ferro-RHP–CO product at 565 and 534 mμ were better resolved than those found with NO, and also differed in appearance.

The responses effected by the cycle of gas changes described above at pH values 5.2, 7.0 and 9.0, were not in evidence at pH 12.0. Thus, the spectrum obtained after incubation in air of the NO products at pH 12.0 showed no structure, and absorbancies in the region 500–600 mμ were lowered significantly relative to the free ferri-RHP spectrum at this pH. Thus, it appeared that changes effected at pH 12.0 were irreversible to a marked extent.

Some observations on the effect of denaturation at pH 7.0 by incubation of RHP with urea (9.0 M) are of interest in connection with the effects of alkali noted above. Thus, ferri-RHP loses its haematin peak at 638 mμ, much as it does at pH 11 in the absence of urea. Moreover, addition of urea to the ferro-RHP–NO products, or CO–ferro-RHP compound at pH 7.0 washes out structure in the spectra to the same extent as incubation at pH 11 or 12 in the absence of urea.

Effect of illumination on RHP–NO compounds

No significant spectral changes were observed at any pH tested when the RHP–NO compounds were illuminated with white light (400 ft candles intensity) for 30 min at 23°. Slight and variable effects were observed with ferro-RHP–NO products. Responses to illumination were examined in detail for the ferrihaemochrome-like product from the oxidized form at pH 5.2 (Product I, see below). It was found that this compound was remarkably stable; no appreciable spectral change was detected even after illumination for 1 h at 1000 ft candles at 23°.

Survey of NO products

In Table I, we present a summary of the spectroscopic properties associated with RHP–NO products formed at the four values of pH tested. It appears reasonable to group these properties under three types, called Product I, Product II, and Product III.

Product I is formed from ferri-RHP at acid or neutral pH and its production is markedly pH-dependent (Fig. 5). Product II appears at all pH values from 5.2 to 9.0 and its formation is essentially pH-independent. Spectroscopically, Products I and II differ in that the ferrohaemochrome-like peaks at 530 and 563 mμ are well-resolved in the spectrum of the former, and the latter exhibits an extra small peak at approx. 480 mμ.

All spectra show isosbestic points at 684, 519 and 410 mμ. Hence, it is possible to calculate amounts of Product I produced from the ratios of absorbancies observed,

either at 563 or 638 mμ, to that for total absorbancy change at pH 5.2; these changes are monitored relative to the reference wavelengths given at the isosbestic points, 519 and 684 mμ, respectively. We find that the decrease in absorbancy at 638 mμ, expressed as percentage of observed change in absorbancy to the total possible, is linearly proportional to the simultaneous absorbancy increase at 563 mμ in the range of pH 5.2 to 7.0.

As seen in Fig. 5 at pH 7, and as remarked previously, approx. 45 % of the ferri-RHP is converted to NO compounds, whereas complete conversion appears to be effected at pH 5.2. The variation in amounts of Product I with pH is shown in Fig. 6.

TABLE I

LOCATION OF ABSORPTION MAXIMA IN SPECTRA OF RHP–NO PRODUCTS AS FUNCTION OF pH

Maxima of which wavelengths are in parentheses are small peaks or humps less prominent than α, β and γ peaks.

Products	*pH* 5.2	7.0	9.0	12.0
		(wavelength in mμ at absorption maxima)		
Oxidized RHP–NO products	*I*	*I*	*II*	*III*
		(638)		
α	563	563	563	565 ~ 570
β	530	530	530	540
		(482)	(482)	(~ 485)
γ	417	417	~ 417	410
			(395)	
Remarks	Characteristic bright pink color	Due to unreacted oxidized form, indicating incomplete conversion to Product I	No sharp separation between α and β peaks	Blurred ferri-haemochrome type, without sharp separation between α and β peaks
Reduced RHP–NO products	*II*	*II*	*II*	*III*
α	~ 560	~ 560	~ 560	565 ~ 570
β	530	530	530	540
	(482)	(482)	(482)	(~ 485)
γ	418	417	417	410
		(395)	(395)	
Remarks	No sharp separation between α and β peaks: color is purplish-pink			Essentially same as formed from oxidized RHP
Reduced RHP–CO compound (in the dark)				
α		565		
β		534 ~ 536		
γ		415		

Similar data for Product II could not be obtained because of the absence of well-defined absorption maxima and the low solubility of this product.

We have found that the spectra in the visible region for Product II at pH 5.2 are easily produced from Product I (initially formed at pH 5.2) by addition, under 1 atm NO, of approx. 5 mg sodium dithionite, or an amount of 10 N KOH solution sufficient to shift the pH from 5.2 to 9.0.

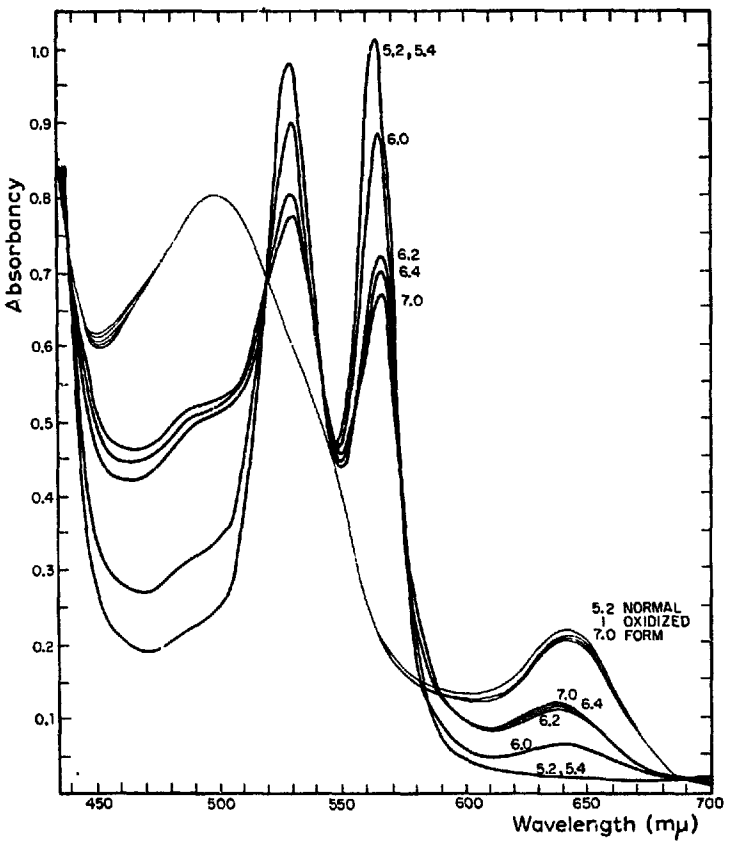

Fig. 5. Effect of pH on formation of the ferri-RHP–NO product (Product I). RHP concentration of 37.8 mμmoles/ml was used. Attached figures indicate pH values. Spectra (5.2) → (7.0) indicate those of normal oxidized RHP at the corresponding pH.

Effect of partial pressure of NO on formation of Product I

The effect of partial pressure of NO on formation of Product I was studied at pH 5.2. The reaction rate for formation was rapid. All spectra produced at various pH values first were monitored 15 min after exposure to NO. Spectra were monitored at least once more after 2 h to establish final stable spectra. Calculation of the percentage of the reacted RHP was made from a set of spectra as shown in Fig. 5 in the same way as described in previous sections. The saturation curve obtained, shown in Fig. 7, indicated that the half-saturation partial pressure for NO at pH 5.2, and 23°, occurred at approx. 0.11–0.15 atm for ferri-RHP. Attempts to obtain corresponding saturation data for the reduced form of RHP at pH 5.2 by measuring its absorbancy at 415 mμ failed, mainly because of uncontrolled effects of the added dithionite, particularly at low pressures of NO. However, it was apparent that the ferro-RHP–NO product (Product II) could be decomposed to some extent by prolonged evacuation more easily than was the case with Product I.

Effect of pH on the amount of the ferro-RHP–CO compound formed in the dark at 1.0 atm CO pressure, and on its photodissociability

Information so far available on the nature of the RHP–CO compound is scanty. Therefore, some experiments were carried out to extend the previous studies[2] and to facilitate comparison with the RHP–NO Products.

It was found first that, despite the previously established absence[4] of detectable changes in the spectra of ferro-RHP itself when pH values changed through the range 4.8–9.3, the intensities of the peak maximum (415 mμ) and minimum (432 mμ) in the spectrum of reduced RHP–CO were affected by pH of the media (Fig. 8). The amount of CO-bound RHP could be estimated by measurement of the absorbancy at 415 mμ in the CO-difference spectrum (reduced RHP–CO minus reduced RHP) corrected for the absorbancy at one of the isosbestic points, *e.g.*, 424 mμ. The minimum absorbancy was found at pH 6.0–6.2. The relative amount of CO compound was plotted against pH by defining the minimum amount determined at pH 6.0–6.2 as 100. As seen in Fig. 9, a considerable increase in formation of the CO compound was observed when the pH was shifted toward or away from 6.0–6.2. Thus, at pH 4.8 or at 9.3, the CO compound formed was approx. 1.2 and 1.25 times higher, respectively, than the minimum amount at pH 6.0–6.2.

Effect of partial pressure of CO on ferro-RHP–CO formation

The effect of the partial pressure of CO on the formation of the ferro-RHP-CO

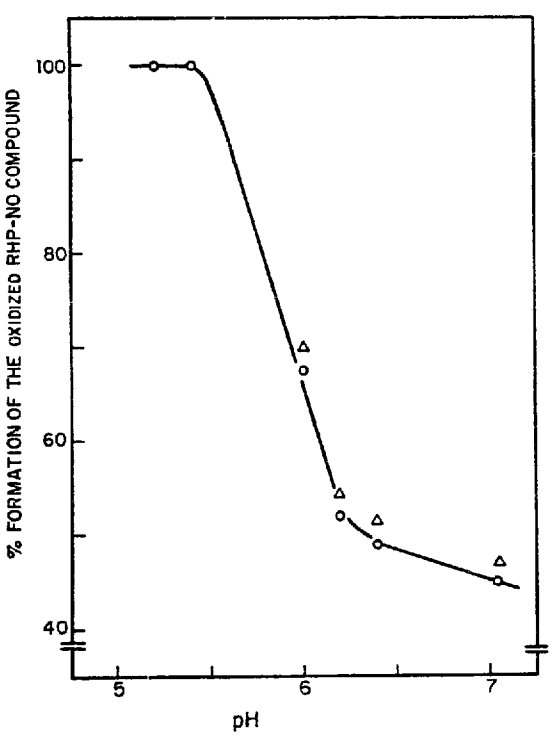

Fig. 6. Plot of the percentage formation of the ferri-RHP–NO compound over the pH range from 5.2 to 7.0. Values were calculated from the data of Fig. 5 as described in the text. O—O, Percentage calculated from the change in absorbancy at 563 mμ; Δ—Δ, percentage calculated from the change in absorbancy at 638 mμ.

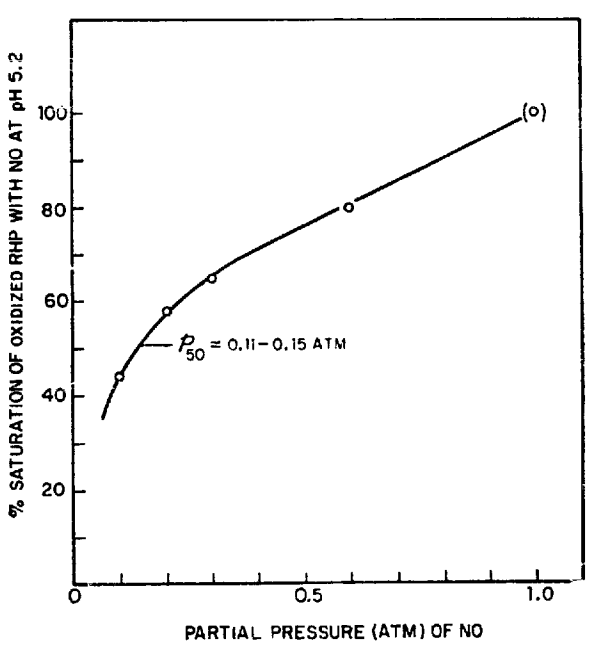

Fig. 7. The equilibrium between RHP and NO at pH 5.2 and 23°. Percentage saturation is plotted against partial pressure of NO. The RHP concentration used for this equilibrium experiment is approx. 37.8 mμmoles/ml.

compound in the dark was examined at four different pH values, 4.8, 6.0, 7.1 and 9.3. The half-saturation values obtained at 23° based, to a first approximation, on the assumption that 100% saturation was attained under 1.0 atm pressure at each pH, were 0.015, 0.013, 0.014 and 0.014 atm, respectively. Thus, it appeared that over this range of pH, RHP exhibited no significant Bohr effect with CO. It should be

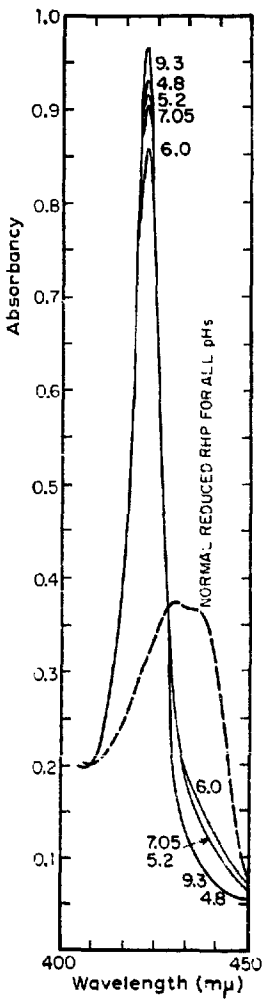

Fig. 8. Effect of pH (4.8–9.3) on the formation of the reduced RHP–CO compound in the dark. RHP concentration of approx. 2.1 mμmoles/ml was used. Figures attached to the spectra indicate pH values.

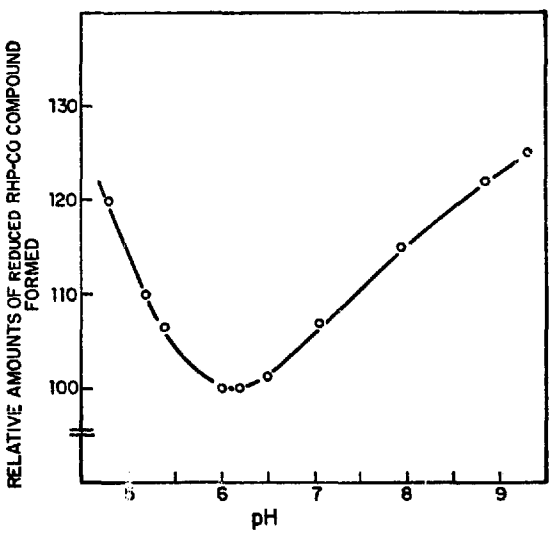

Fig. 9. Percentage formation of the ferro-RHP–CO compound over the pH range from 4.8 to 9.3.

noted, however, that the assumption of 100% was not wholly justified. For example, at pH 7.0 the reduced RHP apparently was not saturated with CO at 1 atm pressure, because when higher pressures (up to 1.5 atm) were used, there was an approximate 8% increase in the absorbancy of the Soret peak (415 mμ) in the ferro-RHP–CO minus ferro-RHP difference spectrum.

Photodissociation of the ferro-RHP–CO compound and its pH dependence

The photodissociable nature of the ferro-RHP-CO compound, a property similar to other CO-binding ferrohaem proteins, and its lessened rate of dissociation at acidic pH have been noted previously[2]. Our data presented in Table II are consistent with these observations. This kind of experiment is qualitative because it is performed without well-defined and quantitated cross-illumination, and because of occurrence of association between CO and ferro-RHP, which starts immediately in the dark and lasts for the approx. 25 sec which intervene between removal of the cuvette from

TABLE II

PHOTODISSOCIATION OF THE REDUCED RHP–CO COMPOUND

Percentage amounts of RHP–CO compound which remain after illumination at 400 ft candles for 30 min at 23° are given. These values are calculated on the basis of the amount of RHP–CO compound formed after incubation for 30 min in the dark at each pH.

Conditions	*pH*			
	4.8	*6.0*	*7.1*	*9.3*
Illuminated under 0.01 atm CO	40	20	20	25
Illuminated after pumping off CO for 1 min	25	10	10	15

the illumination zone and the completion of the spectrophotometric measurement. However, a significant increase in percentage of ferro-RHP–CO compound remaining after illumination is found at pH 4.8, as compared with more alkaline pH values.

DISCUSSION

As seen from Figs. 1–4, measurement of ligand effects are complicated by spectroscopic changes brought about by variations in pH. As noted previously[4] ferri-RHP is relatively insensitive to pH in the range 5.2–7.0, but undergoes drastic changes in spectra at higher values of pH (pH 8–12). Spectra of ferro-RHP exhibit no response to pH change in the alkaline region and but minor changes in acid pH. Taking these changes into account, we find that NO reacts significantly both with ferri- and ferro-RHP over the pH range 5.2–12, while CO reacts in the same pH range only with ferro-RHP. None of the other ligands tested affect the spectra of RHP, either in its oxidized or reduced forms. Taken together with previous researches, the list of unreactive ligands includes: among charged ligands, potassium cyanide, hydroxylamine sulfate, sodium nitrite, sodium azide, sodium fluoride, hydrazine sulfate, and sodium hydrosulfide; among uncharged ligands, nitrosobenzene and 4-methylimidazole. Thus, one primary result is the fact that only small uncharged ligands appear to have access to the haem moieties of *R. rubrum* RHP. We have also ascertained that similar results are obtained with Chromatium RHP*. Hence, we conclude that accessibility only to small uncharged ligands is a general property of the RHP-type haem proteins.

As shown in Table I, the RHP–NO products formed with ferri-RHP are distinguishable from those formed with ferro-RHP and these in turn can be easily distinguished from the CO adduct formed with ferro-RHP. NO, unlike CO, forms not one but at least two different types of product with RHP. The NO–ferri-RHP type exhibits spectra similar to those reported by KEILIN as arising from interaction of NO with free haematins and haem proteins of the open type[8]. Unlike the latter, however, RHP shows less affinity for NO than for CO, although its affinities for either of these ligands are smaller than encountered in typical reactive haem-containing molecules.

There are a number of facts which compel doubt that NO reacts as a true ligand. First, EHRENBERG AND SZCZEPKOWSKI[9] have demonstrated that alkalinization of their

* Kindly donated by Dr. R. G. BARTSCH.

ferri-cytochrome *c*–NO adduct results in recovery of most of the cytochrome in the ferro-form. They rationalize this observation on the basis of a reaction sequence leading to the structure $Fe^{2+}{}_{cytochrome}NO^{+}$. At alkaline pH, they propose this adduct is broken by removal of NO^{+} in reactions with (OH^{-}), *viz*:

$$Fe^{2+}\cdot NO^{+} \rightleftharpoons Fe^{2+} + NO^{+}$$

$$NO^{+} + 2\ OH^{-} \rightleftharpoons NO_2^{-} + H_2O$$

We observe, on the other hand, that only the ferri-RHP type of spectrum results from such a course of procedures, and then only if the pH is maintained below 7–8.

Secondly, we find that Compound I is wholly photostable and that the photostability of Compound II is marginal, if not wholly absent. We expect that true NO-adducts should show some significant degree of photoinstability on the basis of findings such as those of GIBSON AND AINSWORTH[10,11] and KEILIN AND HARTREE[12].

Thirdly, the affinity for NO should be greater than that for CO, inasmuch as NO is universally found to be a more powerful ligand for haematin compounds than is CO[13–15]; in fact, the affinity for NO displayed by RHP is very small and at least an order of magnitude less than that of RHP for CO.

Fourthly, we have attempted to exploit the greater affinity of RHP for CO, relative to NO, to establish the existence of a ferro-haem group in Compound I, by incubation with CO at pH 5.2. Results have been negative, despite our finding that ferro-RHP not exposed to NO at this pH reacts readily with CO. This experiment, as well as the others described, quite clearly forces the conclusion that a typical ligand-bonding reaction, such as that for NO in its interaction with ferricytochrome *c*, does not occur with RHP.

In the case of Product III, which is formed under highly alkaline conditions, irreversible reactions are involved, probably between NO and reactive sites in the peptide chains of RHP, rendered accessible presumably by twisting and distortion of the protein at high alkaline pH. It is evident that major alterations in the structure of RHP occur at pH 12, from the appearance of ferrohaemochrome-type spectra, indicating movement of basic coordinating groups into the coordination sphere of the haem irons. In this condition, the protein is drastically altered by exposure to NO, which causes disappearance of the ferrohaemochrome-type spectrum of alkaline RHP. Nevertheless, no such reactions occur under these conditions when tests are made with charged or large uncharged ligands. These results underscore the essential inaccessibility of the haem groups in RHP, native or denatured, to all but small uncharged ligands. They also bear on the observations made in this laboratory that RHP either from *R. rubrum* or *Chromatium*, undergoes enzymic digestion with trypsin only after drastic heat treatment (K. DUS, private communication).

The pH-dependency of NO-product formation appears to be associated with a haem-linked group of p*K* approximately at pH 6 (Fig. 6). A similar pH-dependence has been described for the fourth haem in haemoglobin in its reactions with NO (see ref. 16). If the amino acid environment in *R. rubrum* RHP is like that found in *Chromatium* RHP (see ref. 17), then we may assume that the group responsible for the pH effect on NO-product formation is the imidazole moiety of histidine.

The pH-dependence found for photodissociation of ferro-RHP–CO in previous researches[2] and confirmed in our present studies appears to be unique to RHP. No such phenomenon has been reported for any other haematin compound.

Our present working hypothesis is that NO, after penetration to the iron-binding sites may act in two ways. First, as a ligand it may give rise to an initial product which, in the case of the ferri-RHP reaction with NO, as in the case of ferricytochrome *c* and NO (see ref. 9), probably involves charge transfer with formation of the NO^+ moiety. We would expect this product to be progressively unstable with increasing pH, as we note is the case (Figs. 5 and 6). The ensuing reactions, after removal of NO^+, would require charge redistribution with partial oxidation to forms which we might expect to occur also when ferro-RHP reacts with NO, with the latter in this case functioning as an oxidant. The product of ferro-RHP and NO (Product II) should not be so pH-unstable as that of ferri-RHP and NO (Product I), a prediction in accord with our findings. The interconvertibility of Products I and II at pH 5.2–7.0, and the formation of identical products from either ferri-RHP or ferro-RHP at pH 9.0 can be rationalized on the same basis. We note, in passing, that the slowness of NO removal from its complexes with ferri- and ferro-haemoglobin, observed many years ago by KEILIN AND HARTREE[18], may be related to similar oxidation–reduction reactions initiated in the haem protein by NO.

The second mode of action may involve an alteration of a labile group which may be a substituent in the chelating porphyrin moiety, or a part of a nearby amino acid residue. The result is formation of a reactive center which can enter into charge-transfer equilibrium with the iron atoms. If the initial reactant is ferri-RHP the eventual product contains iron largely in the ferro state; if RHP reacts in the ferro state, charge transfer is hindered, and reaction to the final ferrohaemochrome-type product is less complete.

It is evident that further clarification requires extension of these studies to include more data, such as may be obtained by measurements of magnetic susceptibility, electron paramagnetic resonance, proton magnetic resonance, and by precision acid–base titrimetry. We may note, however, that the present data definitely contradict the expectation that the alterations in structure of RHP which occur as the result of pH change should lead to ligand interactions. In addition to the demonstration that at pH 5.2–12 only the small uncharged ligands, CO and NO, can penetrate to the prosthetic haem groups of RHP, they further indicate that only the relatively inert CO molecule can express a ligand interaction, because, unlike NO, it does not react with the labile groups in the immediate vicinity of the haem irons. This finding underscores the necessity for the proposed classification of RHP in a new group of haem proteins.

ACKNOWLEDGEMENTS

These researches were supported by grants-in-aid from the National Institutes of Health (C-5992), the National Science Foundation (G-19642), and the C. F. Kettering Foundation, Yellow Springs, Ohio, (U.S.A.).

REFERENCES

1 L. P. VERNON AND M. D. KAMEN, *J. Biol. Chem.*, 211 (1954) 643.
2 R. G. BARTSCH AND M. D. KAMEN, *J. Biol. Chem.*, 230 (1958) 41.
3 *Report of the Commission on Enzymes of the International Union of Biochemistry*, Pergamon Press, Oxford, 1961, Chapter V, I.U.B. Sympos. Series 20.

[4] T. HORIO AND M. D. KAMEN, *Biochim. Biophys. Acta*, 48 (1961) 266.
[5] J. A. ORLANDO, L. LEVINE AND M. D. KAMEN, *Biochim. Biophys. Acta*, 46 (1961) 126.
[6] J. G. ORMEROD, K. S. ORMEROD AND H. GERT, *Arch. Biochem. Biophys.*, 94 (1961) 449.
[7] R. G. BARTSCH AND M. D. KAMEN, *J. Biol. Chem.*, 235 (1960) 825.
[8] J. KEILIN, *Biochem. J.*, 59 (1955) 571.
[9] A. EHRENBERG AND W. SZCZEPKOWSKI, *Acta Chem. Scand.*, 14 (1960) 1684.
[10] Q. H. GIBSON AND S. AINSWORTH, *Nature*, 180 (1957) 1416.
[11] Q. H. GIBSON, *J. Physiol.*, 134 (1956) 112.
[12] D. KEILIN AND E. F. HARTREE, *Biochem. J.*, 61 (1955) 153.
[13] L. HERMANN, *Arch. Anat. Physiol. (Leipzig)*, 469 (1865).
[14] Q. H. GIBSON AND F. J. W. ROUGHTON, *J. Physiol.*, 128 (1955) 69P.
[15] Q. H. GIBSON AND F. J. W. ROUGHTON, *J. Physiol.*, 136 (1957) 507.
[16] Q. H. GIBSON AND F. J. W. ROUGHTON, *Proc. Roy. Soc. London Ser. B*, 147 (1957) 44.
[17] K. DUS, R. G. BARTSCH AND M. D. KAMEN, *J. Biol. Chem.*, 237 (1962) 3083.
[18] D. KEILIN AND E. F. HARTREE, *Nature*, 139 (1937) 548.