A Charge-Transfer Intermediate in the Mechanism of Reduced Diphosphopyridine Nucleotide Oxidation by Ferric Ions*

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ABSTRACT: A blue Fe^{3+}–reduced diphosphopyridine nucleotide complex is formed very rapidly in acid solutions and disappears with first-order kinetics. The complex has two absorption bands at 540 and 375 mμ with extinction coefficients of 900 and about 6000 M^{-1} cm^{-1}, respectively. Stoichiometric titrations and model building studies indicate that bidentate complexing of iron with one, two, and three reduced diphosphopyridine nucleotide molecules is possible. The binding is presumably through the reduced nicotinamide ring nitrogen and N_7 of the adenine . The disappearance of the complex measured at 540 mμ is accompanied by oxidation of reduced diphosphopyridine nucleotide and the reduction of the metal; all these reactions proceed with the same rate constant. The stoichiometry of this redox reaction, after correction for the acid modification of reduced diphosphopyridine nucleotide, is such that reduced diphosphopyridine nucleotide appears as a one-electron donor. Under these conditions oxidized diphosphopyridine nucleotide appears in an equivalent amount to ferrous ion produced. When ferric iron is in high excess or in the presence of flavin mononucleotide, reduced diphosphopyridine nucleotide serves as a two-electron donor. It is concluded that an intramolecular electron transfer occurs from ligand to metal, leading to the formation of an unstable intermediate. This intermediate may undergo disproportionation, or may reduce either ferric ions or flavin mononucleotide. The flavin mononucleotide one-electron reduction product may then transfer this electron to Fe^{3+}. The possibility that this mechanism operates in enzymic oxidations of reduced diphosphopyridine nucleotide is discussed.

At the oxidizing end of the respiratory chain, electrons are transferred from DPNH[1] to an enzymic system, DPNH dehydrogenase, in which FMN, nonheme iron, and labile sulfide are involved (King *et al.*, 1966). There is no clear-cut evidence as to the order in which these components are located in the electron pathway from DPNH to the next electron acceptors of the chain, quinones (Sanadi *et al.*, 1963). Furthermore, it has been established that the respiratory flavins function between their fully oxidized and semiquinoid states (Aleman *et al.*, 1966; Massey *et al.*, 1966). Therefore DPNH dehydrogenase must be the site at which the flow of electrons changes from two-electron to one-electron mechanisms (Beinert and Palmer, 1965).

Nonenzymic model systems in which DPNH reacts directly with FMN were studied by Gascoingne and Radda (1967), Radda and Calvin (1964), Fox and Tollin (1966a,b).

The properties of an alternative nonenzymic model, in which the initial transfer is from DPNH to ferric ions, are presented in this paper. This approach was prompted by the fact that the DPNH molecule contains phosphate groups and basic nitrogens that are potentially good ligands of iron. The studies described were performed at moderately acid pH because of the insolubility of iron in neutral solutions. It was found that a complex between DPNH and ferric ions is formed instantaneously and that it decomposes with simultaneous oxidation of DPNH and reduction of the metal.

The stoichiometry of the reaction of equimolar amounts of DPNH and iron gives a value of 1 equiv of iron reduced/mole of DPNH oxidized. However, in the presence of FMN or of excess of the metal, the stoichiometries observed were in keeping with the values accepted in the literature.

Materials and Methods

Chemicals. DPNH (grade II) was purchased from Sigma Chemical Co.; ferric ammonium sulfate, AR, and silica G were obtained from Merck, Inc.; FMN was a B.D.H. preparation. Twice-crystallized alcohol dehydrogenase was a Boheringer und Soehne preparation. All other reagents were of AR degree of purity.

Methods. Spectrophotometric experiments were performed with a Cary Model 15 instrument; pH values were measured with glass electrodes using a Radiometer PHM4 potentiometer.

Iron was estimated colorimetrically in the reduced state by the *o*-phenantroline procedure (Massey, 1957). DPNH was estimated by two different procedures: spectrophotometrically at 340 mμ or enzymically with alcohol dehydrogenase (Ciotti and Kaplan, 1957). This enzymic assay was also used for the estimation of DPN^+.

The products of DPNH oxidation were separated by thin-layer chromatography on silica G, using water as a developer. DPNH spots were detected by their fluorescence under ultraviolet light. DPN^+ was also detected

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[1] Abbreviations are listed in *Biochemistry 5*, 1445 (1966).

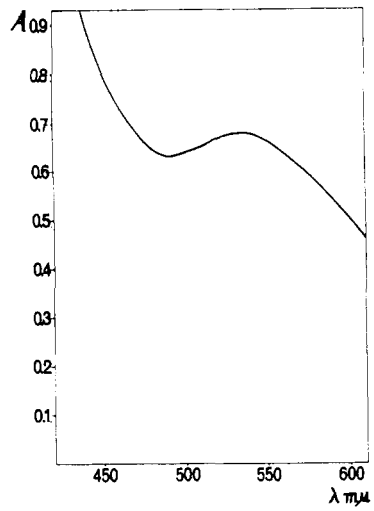

FIGURE 1: Absorbance spectrum of the complex formed between ferric ammonium sulfate (0.78 mM) and DPNH (2.40 mM). Glycine buffer (0.1 M), pH 3.5.

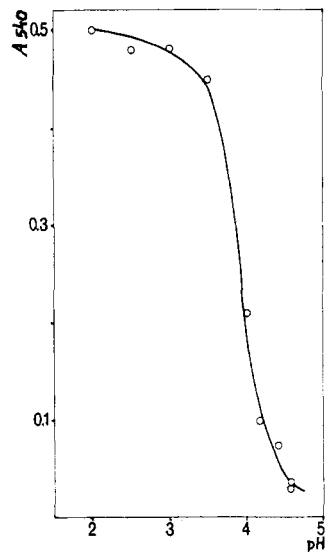

FIGURE 2: The dependence of complex formation upon pH. The complex formation is taken as the zero-time absorbance at 540 mμ. [Fe^{3+}], 0.78 mM; [DPNH], 0.89 mM; 0.1 M glycine or acetate buffers.

by fluorescence, after spraying the plates with 1.0 N sodium hydroxide and drying for 5 min at 100°.

Results

Formation and Properties of Fe^{3+}–(DPNH)$_x$ Complexes. Immediately after the addition of DPNH to a solution of ferric ammonium sulfate in 0.1 M glycine–HCl buffer (pH 3.0), a purple-blue color appeared that faded slowly upon standing. The spectrum of the blue solution between 400 and 700 mμ, shown in Figure 1, is characterized by a single broad band in the visible region, with maximum absorbance at around 540 mμ.

A second band, observed at 375 mμ, was not suitable for kinetic measurements because of the absorbance of DPNH in this region. The normal DPNH absorbance in this region interfered with measurements of the extinction coefficient of this band, and difference spectra could not be used because of the acid modification of DPNH described below. However, by comparison of the absorbancies of the bands at 540 and at 375 mμ, it is possible to assign to the latter an extinction coefficient of between 4000 and 8000 M^{-1} cm^{-1}.

No spectral changes could be detected by mixing ferric ions with DPN^+, or by mixing ferrous ions with either DPN^+ or DPNH.

The shape of the 540-mμ band was independent of the concentrations of either ferric ions or DPNH. Furthermore, when measured immediately after formation at a single pH, the intensity of the band was in a linear relationship to the concentrations of both ferric ions and DPNH.

The intensity of the band was pH dependent as shown in Figure 2. The narrow pH range within which this pH dependence operates, indicates that a single ionization is not sufficient to explain the pH effect. It should be recalled that both the adenine moiety of DPNH and the water molecules solvating the ferric ion undergo ionizations in this region (Hedström, 1953).

The appearance of the 540-mμ band can be best explained by assuming the formation of a ferric iron complex with DPNH as a ligand. In order to investigate the stoichiometry of the complex, it was necessary to measure its absorbance at different concentrations of its components. These measurements were impaired by the fading of the 540-mμ band. The time course of the fading was studied and found to follow first-order kinetics. A typical experiment is shown in Figure 3.

By extrapolating kinetic plots such as those of Figure 3 to zero time it was possible to estimate the initial absorbance of the blue compound. Figure 4 shows the variation of the initial absorbance at 540 mμ with the mole fraction, X_{Fe}, of ferric iron at a given fixed sum of concentrations of both reactants. This type of experiment allows to determine the stoichiometry of a metal–ligand complex using the method of Job (1928).

In the case of Fe^{3+}–(DPNH)$_x$, shown in Figure 4, the intersection of the two lines at $X_{Fe} = 0.35$ indicates the

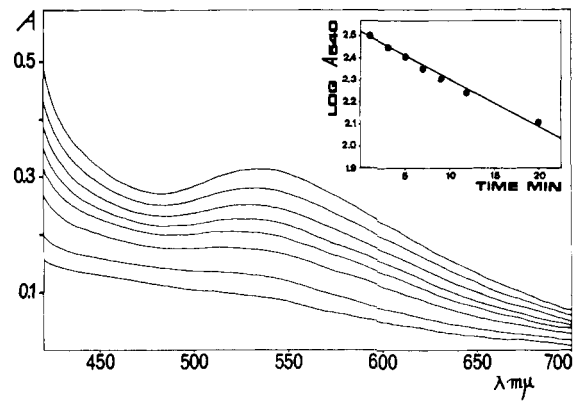

FIGURE 3: Spectra of Fe^{3+}–DPNH complex measured 1, 2, 4, 7, 9, 12, 20, and 30 min after addition of DPNH. [Fe^{3+}], 0.51 mM; [DPNH], 0.91 mM; 0.1 M glycine buffer (pH 3.5). Insert: change in absorbance at 540 mμ with respect to time. Data obtained from the spectra shown in this figure.

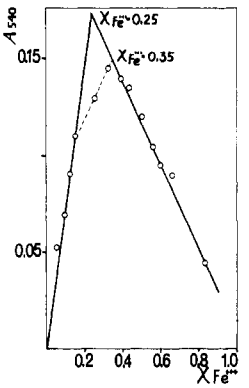

FIGURE 4: The dependence of complex formation upon the mole fraction of ferric iron in solution. The sum of Fe^{3+} and DPNH concentration was 0.56 mM. The absorbancies indicated are the zero-time readings; 0.1 M glycine buffer (pH 3.5).

existence of the complex Fe^{3+}–$(DNPH)_2$. Yet, if only the lowest values of X_{Fe} are used in order to build the left leg of the plot, the intersection point is shifted to $X_{Fe} = 0.25$, indicating a formula Fe^{3+}–$(DPNH)_3$ for the complex. This implies that both di- and tri-DPNH complexes of Fe^{3+} are formed, the latter becoming predominant in high excess of DPNH.

Furthermore, by using a fixed concentration of the metal ion it is possible to titrate the formation of the complex. A typical experiment is shown in Figure 5. At pH 3.5, in a solution 1.08×10^{-4} M in ferric ions, maximal formation of the complex is attained at a DPNH concentration of about 2×10^{-3} M. The zero-time value of the optical density under these conditions is 0.097. Therefore, the extinction coefficient of the blue complex at 540 mμ is about 900 M^{-1} cm^{-1}. Since the total concentration of ferric ions in this titration is of the same order of magnitude of those used in the left portion of the curve in Figure 4, it is plausible to assume that the extinction coefficient estimated above corresponds to the species Fe^{3+}–$(DPNH)_3$.

Oxidation–Reduction Processes in the Fe^{3+}–$(DPNH)_x$ Complexes. It was observed that the disappearance of the 540-mμ band was accompanied by the appearance of ferrous ions and of DPNH oxidation products.

In order to study the relationships among these changes, their kinetics were measured.

As stated above, the 540-mμ band disappeared with first-order kinetics. The rate constants were dependent upon the pH of the solutions, as shown in Figure 6.

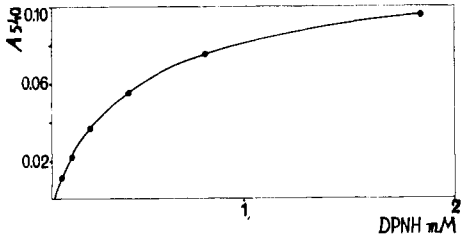

FIGURE 5: Spectrophotometric titration of ferric iron (0.108 mM) with DPNH in 0.1 M glycine buffer (pH 3.5). The absorbancies indicated are the zero-time readings.

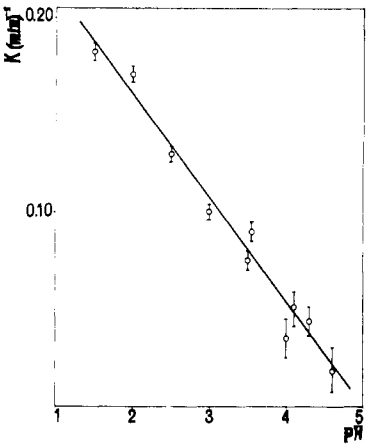

FIGURE 6: The pH dependence of the first-order rate constants of complex disappearance. [Fe^{3+}], 0.78 mM; [DPNH], 0.89 mM; 0.1 M HCl–KCl, glycine or acetate buffers.

It may be seen in Figure 2 that the amount of complex at zero time decreased very rapidly at pH values higher than 3.5. For this reason, pH 3.5 was chosen for the studies described below.

It is known that DPNH undergoes chemical changes in acid solutions (Rafter *et al.*, 1954). This raises the possibility that acid-modified DPNH might be the species involved in the formation of the blue complex. This possibility was excluded by the following experiment. DPNH (220 μM) was kept at pH 3.5 for various periods, at the end of which the absorbance at 340 mμ was measured. This indicated the amount of native DPNH remaining in the solution. Immediately thereafter ferric

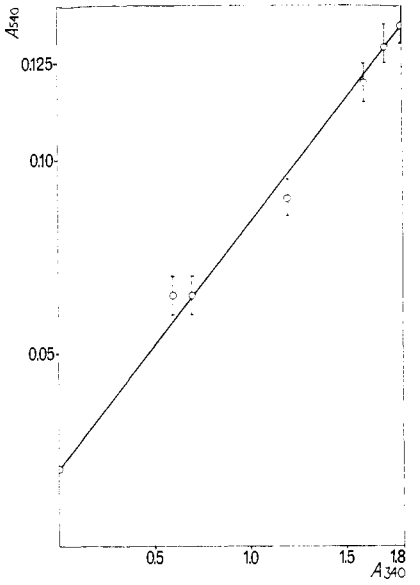

FIGURE 7: The dependence of the Fe^{3+}–DPNH complex formed upon the amount of nonacid modified DPNH in the solution. Absorbancies indicated are zero-time readings. DPNH (0.22 mM) kept for various periods at pH 3.5, 0.1 M glycine buffer. After absorbance at 340 mμ reached desired values, ferric iron was added to final concentration of 0.575 mM. The amount of native DPNH is taken as the absorbance at 340 mμ measured at the time of addition of the ferric iron.

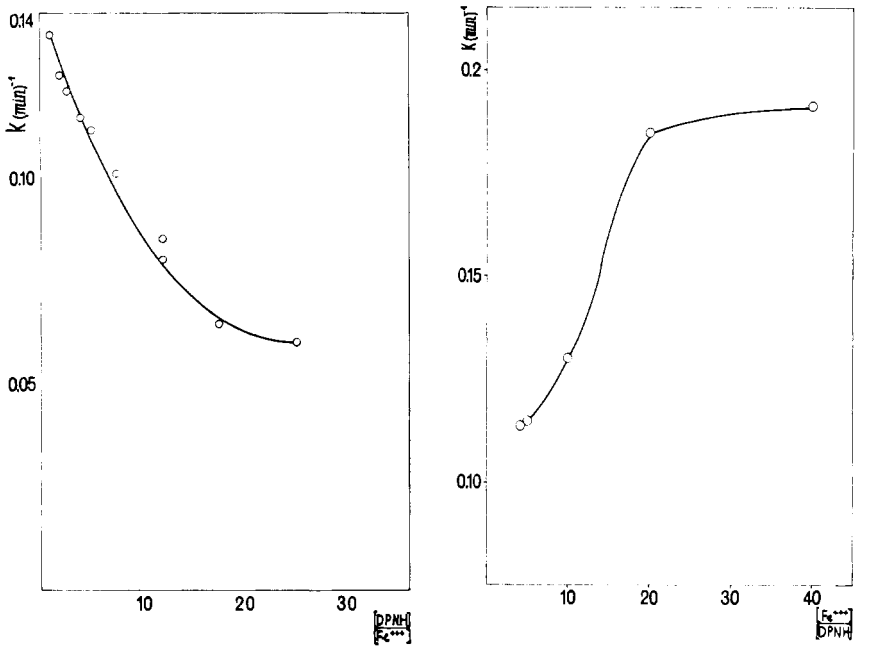

FIGURE 8: The dependence of the rate constant of complex disappearance upon the molar ratio of the reactants in the solution. (a) Left: [Fe^{3+}], 0.400 mM; varying amounts of DPNH. (b) Right: [DPNH], 0.192 mM; varying amounts of Fe^{3+}. The reactions were carried at 25° in 0.1M glycine buffer (pH 3.5).

ammonium sulfate was added to a final concentration of 575 μM. The absorbance was now measured at 540 mμ during a few minutes and extrapolated back to zero time to obtain the initial amount of complex formed. In Figure 7 the amount of complex formed is drawn as a function of the amount of native DPNH remaining in the solution.

The linearity of the graph indicates that only native DPNH participates in the formation of the blue complex.

Experiments were carried out at pH 3.5 in order to compare the rates of complex disappearance, reduction of ferric iron, and oxidation of DPNH. To a solution of 780 μM ferric ions, DPNH was added to a concentration of 890 μM. Aliquots were transferred at different times to 0.1 M ammonium acetate containing 3 mM *o*-phenantroline for the estimation of ferrous ions, and to 0.1 M sodium phosphate buffer (pH 8.3) for the enzymic assay of DPNH. Simultaneously, the disappearance of the 540-mμ band of the blue complex and of the 340-mμ band of DPNH were followed spectrophotometrically.

All the kinetics thus measured were of first order, and their rate constants were the same within their respective experimental errors (Table I). These results indicate that the rate-limiting factor of the oxidation–reduction processes is the concentration of the blue complex, namely, that electron transfer occurs within the complex itself.

It was also observed that for a fixed initial concentration of the metal, the rate constant for the disappearance of the blue complex was lower, the higher the amount of DPNH added (Figure 8a). Conversely, if the initial concentration of DPNH was left fixed the rate constant increased for increasing concentrations of ferric ions until a maximal value was reached (Figure 8b). Since large DPNH/Fe^{3+} ratios favor the formation of Fe^{3+}–$(DPNH)_3$, while large Fe^{3+}/DPNH ratios favor the formation of species with less ligands per metal, both results can be consistently interpreted by assuming that

TABLE I: First-Order Rate Constants Measured for Reactants and Products in the Reaction between Ferric Iron and DPNH.[a]

Component Measured	Method	k (min)$^{-1}$
Complex disappearance	Absorbance at 540 mμ	0.080
Fe^{2+} appearance	Reaction with *o*-phenantroline	0.087
DPNH disappearance	Absorbance at 340 mμ	0.078
DPNH disappearance	Enzymic oxidation	0.085

[a] Reaction conditions: 25°, [Fe^{3+}] 0.78 mM, [DPNH] 0.89 mM, 0.1 M glycine buffer, and at pH 3.5.

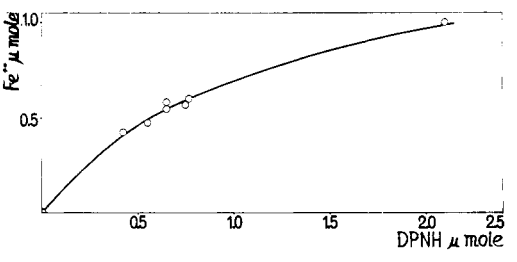

FIGURE 9: The amounts of ferrous iron formed upon reducing 1.120 μmoles of ferric iron by various amounts of DPNH; 0.1 M glycine buffer (pH 3.5).

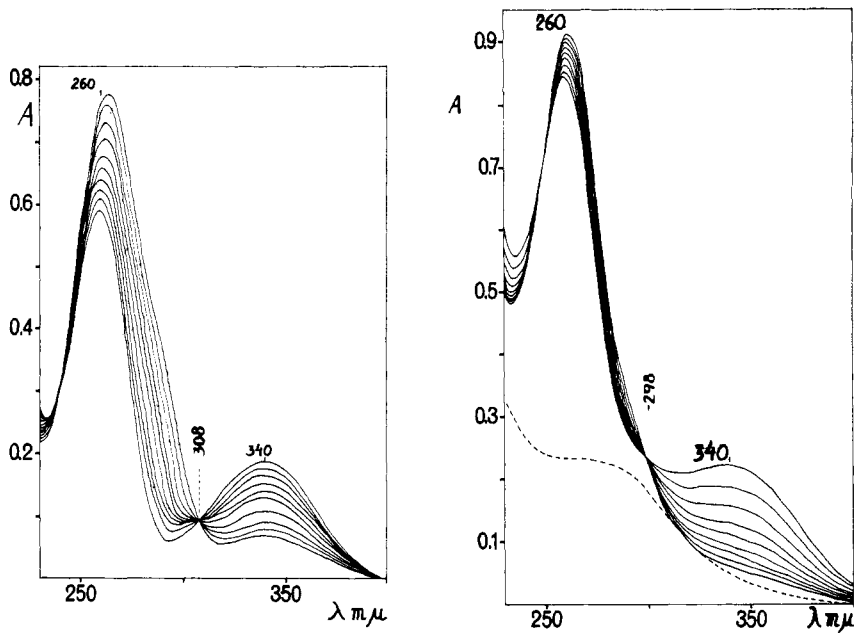

FIGURE 10: Repeated scanning of DPNH solution during acid modification at room temperature in 0.1 M glycine buffer (pH 3.5). Spectra were measured after various periods up to 20 min from the addition of DPNH. Scanning time, 13 sec. (a) Left: spectral changes of 29 μM DPNH. (b) Right: spectral changes of 29 μM DPNH in presence of 75 μM ferric iron. The dotted line is the spectrum of the ferric iron alone.

in the complexes with higher degree of ligation, the electron transfer is slowest.

The stoichiometry of the oxidation of DPNH by ferric ions was studied in a small excess of the latter. At the end of the reaction the amount of ferrous ions formed was measured and compared with the known amount of DPNH initially added.

It was observed that there was no simple correlation between the number of microequivalents of ferrous ions formed and the number of microequivalents of DPNH consumed in the reaction (Figure 9). The same results were obtained when the experiments were performed in atmospheres of air, hydrogen, or nitrogen, indicating that the anomaly observed was not due to autooxidation of DPNH. However, since experiments showed that acid-modified DPNH did not reduce ferric ions,

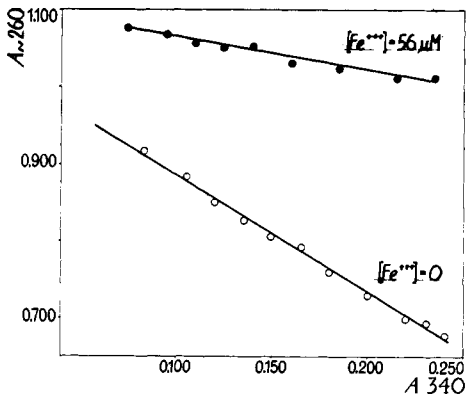

FIGURE 11: The relation between the variations in absorbance at ~260 and 340 mμ during acid modification of DPNH in 0.1 M glycine buffer (pH 3.5). The concentration of ferric iron is indicated in the figure.

the fact that DPNH was exposed to pH 3.5 during the reaction had to be taken into account. To correct for this factor, the spectral changes that accompany acid modification of DPNH were studied at pH 3.5 in the presence and in the absence of ferric ions. When ferric ions were absent, the acid modification of DPNH was characterized by the decrease of the 340-mμ absorption and the simultaneous increase and small red shift (6–8 mμ) of the 260-mμ band (Figure 10a). The behavior of the 340-mμ band was the same when ferric ions were present, but the increase of the 260-mμ band and its red shift were markedly smaller (Figure 10b).

Figure 11 shows the linear relationship between the changes at 260 and 340 mμ in the presence and absence of ferric ions (lines I and II, respectively). The decrease in absorbance at 340 mμ upon oxidation of DPNH is well known. Thus, the observed decrease of 340 mμ in the presence of iron may be taken as the additive result of acid modification and oxidation. However, from the slope 1.6 of line I (Figure 11), the effect of acid modification alone may be estimated from the expression $\Delta A_{(340\ m\mu)} = \Delta A_{(260\ m\mu)}/1.6$. Using this value it was possible to estimate the fraction of total DPNH that was oxidized by Fe^{3+} ions, from the equation

$$\text{fraction of DPNH oxidized} = \frac{\Delta A_{(340\ m\mu)} - \frac{\Delta A_{(260\ m\mu)}}{1.6}}{\Delta A_{(340\ m\mu)}}$$

Results of these calculations are shown in Figure 12.

The data given in Figure 9 refer to the total amount of DPNH consumed in the reaction by oxidation and acid modification. The fraction consumed by oxidation was estimated from Figure 12; the total amount of

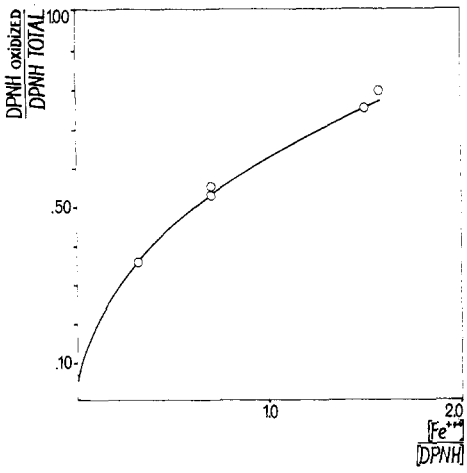

FIGURE 12: The dependence of the fraction of DPNH oxidized by ferric iron upon the molar ratio between the reactants. DPNH (29 μM) was reacted with various amounts of ferric iron and the fraction of DPNH oxidized was calculated (see text); 0.1 M glycine buffer (pH 3.5).

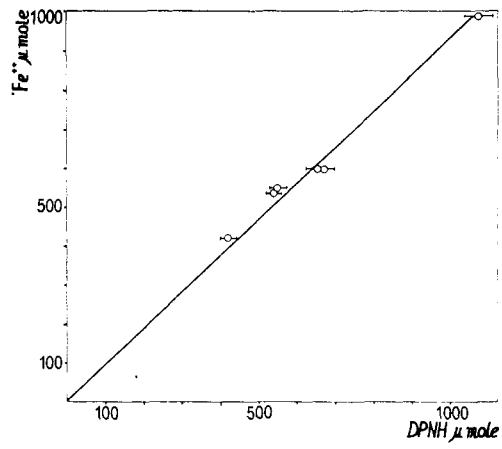

FIGURE 13: The amounts of ferrous iron formed by reduction of ferric iron by various amounts of DPNH. These are the same data appearing in Figure 9, but corrected for acid modification of DPNH as explained in the text. The correction factors were estimated from Figure 12.

DPNH consumed, multiplied by the fraction consumed by oxidation, gave the actual amount of DPNH oxidized by Fe^{3+} ions. The data of Figure 9, corrected in this way, are shown in Figure 13. In this case a linear relationship is obtained, with a slope of about 1.0. This corresponds to a stoichometry of 1 equiv of Fe^{3+} ions reduced by 1 mole of DPNH.

This is in obvious disagreement with the known fact that DPNH is a two-electron donor. Therefore, one should expect that in reactions having the unusual stoichiometry described. the oxidation of DPNH should not lead to DPN^+. Yet, when DPNH was allowed to react with a small excess of ferric ions, the same number of equivalents of DPN^+ and ferrous ions were obtained.

Attempts were made to obtain conditions under which DPNH is oxidized to DPN^+ by 2 equiv of ferric ions. These attempts were successful in two cases. First, when large excesses of ferric ions were present in the reaction mixture, the number of equivalents of ferric ions reduced per mole of DPNH consumed increased, reaching the normal value of 2.0 for a molar ratio, $[Fe^{3+}]/[DPNH] = 40$. Second, in the presence of 0.12 mM FMN, the normal stoichiometry was reached at the markedly lower molar ratio, $[Fe^{3+}]/[DPNH] = 8$. These results are shown in Figure 14. It must be emphasized that at the large Fe^{3+} ion concentrations used in these experiments there is practically no acid modification of DPNH, as seen in Figure 12. Finally, in the products of these reactions with normal stoichiometry, DPN^+ could be identified by thin-layer chromatography with the same R_F as DPN^+ added as a marker.

Discussion

The Nature of the Fe^{3+}–DPNH Complex. Two kinds of groups of the DPNH molecule are best fit to act as ligands of Fe^{3+} ions: the ionized phosphate hydroxyls and the nitrogens of both rings. The first are ruled out from our considerations because ferric ion complexes with either phosphate ions or adenosine triphosphate are colorless (Goucher and Taylor, 1964), while the Fe^{3+}–DPNH complex is blue. Thus, we may conclude that in the complex described here there is bonding between the Fe^{3+} ions and some of the nitrogens of both rings. At pH values below 3.5, where formation of the complex is maximal, the amino group of adenine is fully protonated and cannot function as a metal ligand.

The involvement of the nicotinamide ring in this bonding is sustained by several facts. First, the nicotinamide group, directly affected by the acid modification of DPNH (Rafter *et al.*, 1954), is protected from this attack by iron binding (Figure 10); second, DPN^+ does not form a similar complex, while differing chemically from DPNH only in its nicotinamide moiety; third, the formation of the complex is followed by the simultaneous reduction of the metal and oxidation of the ligand to DPN^+, which requires direct bonding between the Fe^{3+} cation and the electron donating group, reduced nicotinamide.

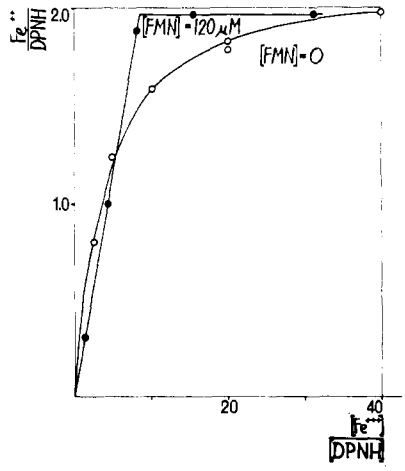

FIGURE 14: The yield of moles of ferrous iron formed per mole of DPNH oxidized measured at various ratios of iron to DPNH; 0.1 M glycine buffer (pH 3.5). [DPNH], 0.120 mM; (●—●) [FMN], 0.120 mM; (O—O) no FMN.

Based on these assumptions, it was possible to build molecular models of Fe^{3+}–DPNH and Fe^{3+}–$(DPNH)_2$, using space-filling models. In those models, the ferric ion is shown to be coordinated octahedrally by the nicotinamide ring nitrogens and by N_7 of the adenines, the two rings of a same DPNH molecule being located either in *trans* positions, or with an angle of 90° for the N–Fe–N bonds. Thus, from a sterochemical point of view, these structures are entirely plausible. On the other hand, a Fe^{3+}–$(DPNH)_3$ complex could be built only with the nicotinamide ring nitrogens as ligands using tetrahedral coordination for the metal.

The 540-mμ band of the complex could result from a number of factors. First, the possibility of a charge-transfer transition from ligand to metal orbitals should be considered. Examples of such assignments are the iron complexes described by Williams (1959). However, the complex decomposes with simultaneous electron transfer, indicating high probability for the completion of the charge-transfer transition, whereas the extinction coefficient of the 540-mμ band is too low to represent a transition with high probability. In the second place, the band may arise from a DPNH free radical similar to the pyridinium-type radicals described by Kosower (1965). However, this would require that ferrous ions appear in solution together with the appearance of the blue color, since there is no other electron acceptor in the solution than ferric ions. Our kinetic experiments show that at zero time, when the blue complex has maximal concentration, the concentration of ferrous ions is nil, ruling out pyridinium-type radicals as the blue chromophores. Finally, the 540-mμ band could represent a d–d transition. Such transitions are Laporte forbidden, but in this case it may become allowed because everyone of the possible complexes lacks a center of symmetry. The position of the band indicates splitting of the iron d orbitals slightly smaller than in the case of ferricyanide. This is in keeping with the expected location of ring nitrogen ligands in the spectrochemical series, as well as with the smaller d orbital splitting in tetrahedral than in octahedral coordination. Furthermore, the intensity of the band is very close to that of the 420-mμ band of ferricyanide (ϵ 1.04×10^3 $M^{-1}\,cm^{-1}$).

The identical rates of disappearance of the blue complex, appearance of ferrous ions, and disappearance of DPNH indicate that electron transfer from DPNH to ferric iron occurs within the complex. This behavior is characteristic of charge-transfer complexes, and the one described here fits within this class, from the point of view of its chemical behavior. From the spectroscopic point of view, the blue complex fulfills as well the requirement of an absorption different from that of either donor or acceptor (Kosower, 1966). The high intensity of the band at 375 mμ is consistent with a charge transfer origin.

As indicated above, the extinction coefficient of the complex in excess of DPNH is 900 $M^{-1}\,cm^{-1}$ (Figure 5). It is shown from the titration in Figure 4 that under these conditions the species Fe^{3+}–$(DPNH)_3$ predominates in the system. At the other extreme, when the complex is formed in large excess of iron, it may be assumed that Fe^{3+}–$(DPNH)_3$ is the only component observed. From Figure 7 one can estimate the ratio between the absorbancies of 540 and 340 mμ, corresponding to the complex and the free ligand, respectively, at the same concentration of DPNH. This ratio is the slope of the line in Figure 7 which has a value of 0.05. For DPNH, $\epsilon_{340\ m\mu}$ is $6.22 \times 10^3\ M^{-1}\,cm^{-1}$. Therefore, for Fe^{3+}–(DPNH) we estimate $\epsilon_{540\ m\mu}$ at $310\ M^{-1}\,cm^{-1}$.

If addition of further identical ligands to Fe^{3+} ions causes additive increases of the extinction coefficients of the resulting complexes, one should expect for Fe^{3+}–$(DPNH)_3$ a value of ϵ $930\ M^{-1}\,cm^{-1}$, in close agreement with the value estimated from Figure 5.

If the data of the titration of Fe^{3+} ions with DPNH are drawn in a Hill plot, $\log x/(1-x)$ *vs.* log [DPNH], where x represents the fraction of metal combined with ligand, a straight line with a slope of 1.0 is obtained. This may be taken as an indication that there is no observable interaction between the metal-bound ligands. The resulting titration constant of Fe^{3+}–$(DPNH)_3$ is about 1.4×10^4 M.

The Mechanism of Reduction of Fe^{3+} Ions by DPNH. The first-order kinetics of the redox reactions and complex disappearance suggest that the rate-limiting step of electron transfer is a unimolecular reaction; this is obviously identified with the completion of the charge-transfer transition.

Although the first-order rate constants are independent of the concentration of the complex, they depend upon the initial ratio of ligand to metal. The higher the ligand to metal ratio, the lower the rate constant; therefore, Fe^{3+}–$(DPNH)_3$ appears as the less labile complex, while electron transfer is faster in less ligated species. We have no explanation for this observation.

Another important fact to be considered is the effect of the initial ratio of ligand to metal concentration on the over-all stoichiometry of the process and the nature of the products. In the excess of DPNH or in small excess of Fe^{3+} ions, 1 g-ion of Fe^{2+} ions appears together with the disappearance of 1 mole of DPNH; in other words, only half of the reducing equivalents of DPNH are consumed in the reaction. However, the number of equivalents of DPN^+ formed equals the number of equivalents of Fe^{2+} ions produced. The question rises as to the fate of the additional equivalent of DPNH that is consumed but does not appear as DPN^+. A mechanism that explains these facts is the following. Within the complex, formed in a very rapid reaction

$$\text{DPNH} + \text{Fe}^{3+} \underset{\text{rapid}}{\overset{\text{very}}{\rightleftharpoons}} \text{Fe}^{3+}\text{–DPNH} \qquad (1)$$

A ligand electron is transferred to the metal in the rate-limiting step

$$\text{Fe}^{3+}\text{–DPNH} \xrightarrow[\text{limiting}]{\text{rate}} \text{Fe}^{3+}\text{–DPNH} \qquad (2)$$

This is followed by the immediate decomposition of the complex, into ferrous ions and a one-electron oxidation product of DPNH, that we symbolize as $DPNH^+$.

$$Fe^{2+}\text{–}DPNH^{+} \longrightarrow DPNH^{+} + Fe^{2+} \quad (3)$$

The species $DPNH^{+}$ must possess free-radical nature and react very rapidly. A typical reaction for the $DPNH^{+}$ free radical is its disproportionation

$$2DPNH^{+} \longrightarrow DPNH + DPN^{+} + H^{+} \quad (4)$$

This reaction has already been suggested in the literature (Isenberg *et al.*, 1961). The DPNH molecules formed in reaction 4 are not protected from acid modification by ferric ions, since this mechanism operates in excess of DPNH, and therefore they disappear rapidly without concomitant formation of DPN^{+}. Thus, 1 mole of DPNH is consumed, while only 1 equiv reacts in the reduction of Fe^{3+} ions. It should be emphasized that the correction for acid modification of DPNH derived from Figure 12 relates only to the DPNH that is not iron bound at the beginning of the reaction; the molecules of DPNH freed from their iron complex upon reduction of the metal are those deprived from protection against acid modification.

When the metal is in large excess, DPNH acts as a two-electron donor as in its common biological reactions. This requires for the $DPNH^{+}$ species to react further with the oxidized iron. One possible pathway for this additional reaction is a direct electron transfer

$$DPNH^{+} + Fe^{3+} \longrightarrow DPN^{+} + H^{+} + Fe^{2+} \quad (5)$$

Another possible pathway in the formation of blue complex from the excess metal and the DPNH formed in reaction 4. In this case, the effect of excess iron in the over-all stoichiometry should be due to the protection afforded by the metal, and the curve in Figure 12 and curve a in Figure 14 should be identical. This, however, is not the case, and we prefer the mechanism that includes reaction 5.

Finally, the presence of flavin can substitute for the presence of excess iron in the system (Figure 14). This behavior strongly suggests that the role of flavin in the mechanism described above is to accept an electron from $DPNH^{+}$ and transfer it to ferric iron (reactions 6 and 7).

$$DPNH^{+} + FMN \longrightarrow DPN^{+} + FMNH^{\cdot} \quad (6)$$

$$FMNH^{\cdot} + Fe^{3+} \longrightarrow FMN + Fe^{2+} + H^{+} \quad (7)$$

In such a mechanism flavin reacts between its oxidized and semiquinoid states, as it reacts in fact in biological systems. Hemmerich and Spence (1966) showed the presence of a flavin free-radical signal in mixtures of flavin and ferrous ions. They attributed this signal to a flavin–ferrous iron charge-transfer complex. Our mechanism requires the reaction between a flavin free radical and ferric iron, and the transition state of this reaction should be identical with the free radical described by Hemmerich and Spence (1966). Experiments to test for this possibility are presently being undertaken.

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