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Phenyldiimide, Hemoglobin, and Heinz Bodies

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Abstract. Phenylhydrazine is oxidized stoichiometrically by two equivalents of ferricyanide to produce phenyldiimide and ferrocyanide. This reaction permits accurate determination of the absorption maxima and molar absorption coefficients of phenyldiimide in aqueous solution. These values are identical or extremely similar to those of phenyldiimide produced by decarboxylation of phenylazoformic acid. Knowledge of the stoichiometry of the reaction confirms an earlier suggestion that phenyldiimide is the product of ferricyanide-oxidized phenylhydrazine that reacts with heme proteins. Homologous structures postulated for phenyldiimideferrihemoglobin and nitrosobenzeneferrohemoglobin suggest a common factor in the production of Heinz bodies after exposure of erythrocytes to phenylhydrazine and to arylamines.

Phenyldiimide, $C_6H_5N{=}NH$, has long been believed to be produced as an unstable intermediate compound in the oxidation of phenylhydrazine to benzene and nitrogen. Chattaway[1] mentioned this possibility in his study of the oxidation of phenylhydrazine by oxygen, and Rekasheva and Mikluklin[2] postulated the intermediate formation of phenyldiimide in the oxidation of phenylhydrazine by potassium ferricyanide. Recently, phenyldiimide has been isolated as a product of electrochemical and cupric chloride oxidation of phenylhydrazine.[3,4] Phenyldiimide is also produced in the decomposition of certain phenylazo compounds. Widman[5] postulated tribromophenyldiimide to be an unstable intermediate in the decomposition of 2,4,6-tribromophenylazoformic acid to tribromobenzene, nitrogen, and carbon dioxide; and Cohen and Nicholson[6] concluded that phenyldiimide is produced in the methanolysis of *N*-phenyl-*N'*-benzoyldiimide. Huang and Kosower[7] showed that phenyldiimide can be produced by the decarboxylation of phenylazoformic acid and reported the visible and ultraviolet spectra of the unstable product.

The work to be reported here has shown that oxidation of phenylhydrazine by ferricyanide is a stoichiometric reaction, and that the only products of the initial reaction are ferrocyanide and phenyldiimide. Because of the instability of the latter compound in water, accurate data on its spectral properties in aqueous solution are not available. Knowledge of the stoichiometry of the phenylhydrazine-ferricyanide reaction has permitted reproducible determinations of the spectral constants of the oxidation product of phenylhydrazine. The same constants of the decarboxylation product of phenylazoformic acid were also determined. Wavelengths of the three absorption maxima of the two products

in the ultraviolet and visible regions were found to be identical, and the molar extinction coefficients found in this study were nearly the same. Evidence was obtained that the initial product of oxidation of phenylhydrazine by oxygen also is phenyldiimide. By showing that phenyldiimide is the stoichiometric product of the oxidation of phenylhydrazine by ferricyanide, these results confirm an earlier suggestion that the ligand of heme proteins in ferricyanide-oxidized phenylhydrazine is phenyldiimide.[8] A structure is postulated for the compound of phenyldiimide with ferrihemoglobin, and a possible role of phenyldiimide in the formation of Heinz bodies is postulated.

Materials and Methods. Phenylhydrazine hydrochloride (Eastman $C_6H_5NHNH_2 \cdot HCl$) was recrystallized 2 or 3 times from ethanol. Potassium ferrocyanide (Baker and Adamson reagent grade $K_4Fe(CN)_6 \cdot 3H_2O$) was dried to constant weight and analyzed for nitrogen (calcd., 22.82%; found 22.85%). Potassium ferricyanide (Baker reagent grade $K_3Fe(CN)_6$, assay 100.0%) was used as obtained. Methyl phenylazoformate (Calbiochem B grade) was used as obtained (calcd., C 58.53%, H 4.91%, N 17.06%; found, C 58.48%, H 4.98%, N 16.78%).

Absorption spectra were recorded with a Cary model 14 spectrophotometer. In order to obtain mixtures of phenylhydrazine and its oxidation product, an excess of phenylhydrazine hydrochloride was treated with increasing concentrations of potassium ferricyanide. The ultraviolet spectrum of each resulting solution was measured against a blank solution that contained the same concentration of potassium ferrocyanide as the concentration of potassium ferricyanide added to the reaction mixture. In other experiments, the concentration of phenylhydrazine calculated to be in excess in the reaction mixture was also added to the blank solution. These reagents were weighed out into volumetric flasks to make solutions of concentration 0.05 to 0.2 M in phosphate buffer of pH 7.34, μ 0.167, and accurately measured volumes of these solutions were delivered with the use of Hamilton microliter syringes (Hamilton Company, Whittier, Calif.) into the cuvette that was used both as the reaction vessel and absorption cell. The decarboxylation product of phenylazoformic acid was generated by saponifying weighed amounts of methyl phenylazoformate in volumetric flasks with 0.6 N NaOH and adding the resulting solution of sodium phenylazoformate to phosphate buffers near neutral pH.[7] Data for extinction coefficients were obtained by taking continuous recordings of absorbance at the wavelengths of absorption maxima; such readings were begun 20 sec after phenyldiimide was generated by either method. The resulting tracings were extrapolated to zero time to obtain initial absorbances. The calculated absorbance of excess phenylhydrazine was subtracted from the absorbance of mixtures of phenylhydrazine and its oxidation product. Oxygen was excluded in these experiments by the use of 99.995% nitrogen passed through a solution of vanadyl sulfate.[9] Reactions were conducted in a square silica cuvette of 10 $\times$ 10 mm cross section to which an open neck equipped with a sidearm was fused. A three-way stopcock was attached to the sidearm. Nitrogen was bubbled through 4.00 ml of phosphate buffer (pH 7.34, μ 0.167) in the cuvette through a long needle, and was at the same time passed into the neck of the cuvette through the sidearm. After the reactants were added and mixed under nitrogen, the needle was withdrawn, and the neck of the cuvette was stoppered while introduction of nitrogen through the sidearm was continued. The stopcock was then closed. The reaction of phenylhydrazine with oxygen was also studied; oxygen was passed through a solution of phenylhydrazine for 30 sec and the spectrum was recorded periodically.

Results. Solutions of partially oxidized phenylhydrazine were prepared by mixing potassium ferricyanide and phenylhydrazine hydrochloride in molar ratios of less than 2:1; if the molar concentration of ferrocyanide was C_f, and the molar concentration of phenylhydrazine was C_p, then C_f was less than $2C_p$. The molar concentration of potassium ferrocyanide in the blank cell was C_f. The

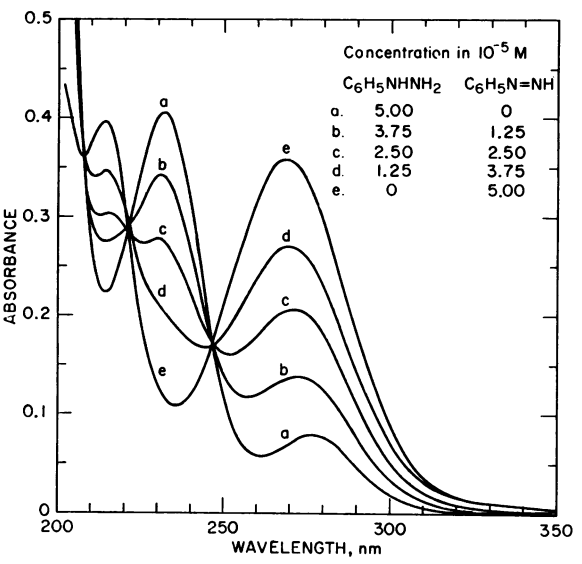


FIG. 1. Ultraviolet spectra of phenylhydrazine and phenyldiimide in phosphate buffer of pH 7.34, μ 0.167. Path length, 1 cm. (*a*) 5.00 × 10^{-5} M $C_6H_5NHNH_2$, (*b*), (*c*), and (*d*) 5.00 × 10^{-5} M $C_6H_5NHNH_2$ reacted with 2.50, 5.00, and 7.50 × 10^{-5} M $K_3Fe(CN)_6$. Spectra were taken with reaction mixture in the sample cell and $K_4Fe(CN)_6$ in the blank cell. The concentration of $K_4Fe(CN)_6$ in the blank cell was the same as the original concentration of $K_3Fe(CN)_6$ in the sample cell. Each spectrum represents a different reaction mixture, and the spectra were recorded within 3 min after the reagents were mixed. (*e*) 5.00 × 10^{-5} M $C_6H_5N=NH$. 10.00 × 10^{-5} M $C_6H_5NHNH_2$ and 10.00 × 10^{-5} M $K_3Fe(CN)_6$ were reacted in the sample cell. 5.00 × 10^{-5} M $C_6H_5NHNH_2$ and 10.00 × 10^{-5} M $K_4Fe(CN)_6$ were in the blank cell.

spectra of several such solutions in which C_p was kept constant and C_f was varied were recorded, and Fig. 1 shows one of the series of spectra. Isosbestic points were found at 207, 222, and 247 nm. The same isosbestic points were observed initially when a solution of phenylhydrazine was exposed to oxygen (Fig. 2). The concentrations of unreacted phenylhydrazine in the reaction mixtures were calculated by assuming that a half mole of phenylhydrazine was oxidized per

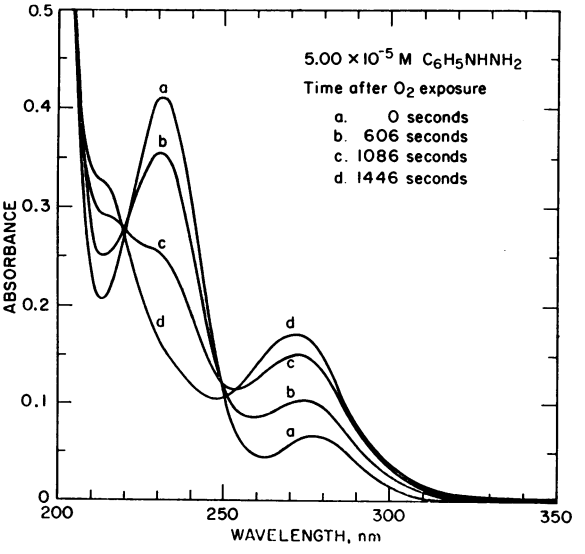


FIG. 2. Ultraviolet spectrum of 5.00 × 10^{-5} M $C_6H_5NHNH_2$ in phosphate buffer of pH 7.34, μ 0.167 before and after exposure to oxygen. Path length, 1 cm. After the spectrum of phenylhydrazine was recorded, oxygen was bubbled through the solution for 20 sec. Times at which absorbance at 270 nm was recorded are shown. Secondary reactions such as decomposition of phenyldiimide and oxidation of phenyldiimide by oxygen cause deviation from isosbestic points after the initial phase of the oxidation of phenylhydrazine by oxygen.

mole of ferricyanide added. When this concentration of phenylhydrazine ($C_p - {}^1/_2\, C_f$) was added to the blank cell, the resulting molar extinctions were independent of the molar ratio of ferricyanide to phenylhydrazine as long as phenylhydrazine was present in excess.

Sodium phenylazoformate was decarboxylated by mixing with phosphate buffer in an oxygen-free cuvette, and spectra were recorded against buffer blanks. Figs. 3 and 4 show the ultraviolet spectra of the oxidation product of phenylhydrazine and the decarboxylation product of phenylazoformate, respectively. Figs. 5 and 6 show the visible spectra. The molar extinction coefficients at the absorption maxima of the two products are shown in Table 1.

TABLE 1. *Absorption maxima* (λ_{max}) *and molar extinction coefficients* (ϵ_M) *at* λ_{max} *of phenyldiimide in phosphate buffer, pH 7.34,* μ *0.167.**

	$\epsilon_M \pm$ S.E. (no. of detns.) of $C_6H_5N{=}NH$ from	
λ_{max} (nm)	$C_6H_5NHNH_2$	$C_6H_5N{=}NCOOH$
214	7940 ± 150 (8)	7740 ± 70 (9)
269.5	7290 ± 30 (8)	7050 ± 10 (9)
400	150 ± 1 (9)	147 ± 1 (9)

* Reported values[7] of λ_{max} (ϵ_M), in this buffer, of phenyldiimide from $C_6H_5N{=}NCOOH$ are 214 nm (10,000), 270 nm (7400), and 401.5 nm (160).

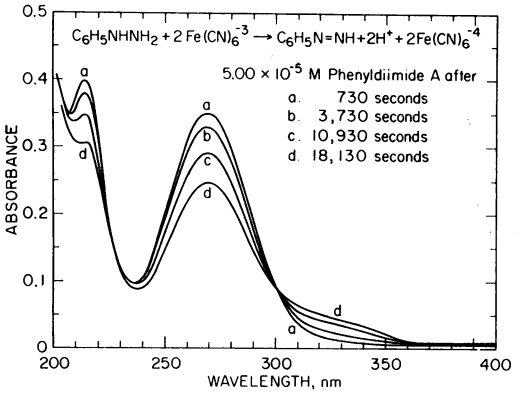


FIG. 3. Ultraviolet spectrum of the oxidation product of phenylhydrazine (phenyldiimide A) in phosphate buffer of pH 7.34, μ 0.167. Path length, 1 cm. Times at which absorbance at 270 nm was recorded are shown. 10.00×10^{-5} M $C_6H_5NHNH_2$ and 10.00×10^{-5} M $K_3Fe(CN)_6$ were reacted in the sample cell. 5.00×10^{-5} M $C_6H_5NHNH_2$ and 10.00×10^{-5} M $K_4Fe(CN)_6$ were in the blank cell.

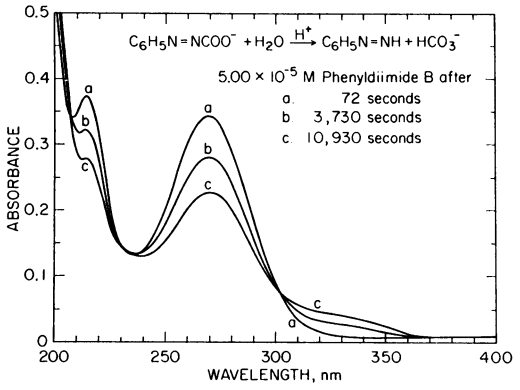


FIG. 4. Ultraviolet spectrum of the decarboxylation product of phenylazoformic acid (phenyldiimide B) in phosphate buffer of pH 7.34, μ 0.167. Path length, 1 cm. Times at which absorbance at 270 nm was recorded are shown. Saponified methyl phenylazoformate (5.31×10^{-3} M in 0.6 N NaOH) was added to phosphate buffer to yield 5.00×10^{-5} M $C_6H_5N{=}NCOO^-$. A volume of 0.6 N HCl equal to the volume of 0.6 N NaOH used was added in order to minimize pH change.

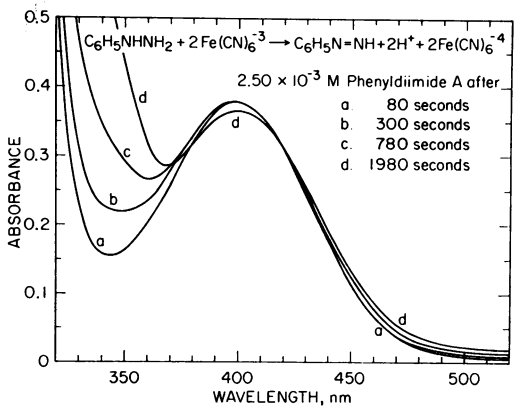


FIG. 5. Visible spectrum of the oxidation product of phenylhydrazine (phenyldiimide A) in phosphate buffer of pH 7.34, μ 0.167. Path length, 1 cm. Times at which absorbance at 400 nm was recorded are shown. 5.00×10^{-3} M $C_6H_5NHNH_2$ and 5.00×10^{-3} M $K_3Fe(CN)_6$ were reacted in the sample cell. 2.50×10^{-3} M $C_6H_5NHNH_2$ and 5.00×10^{-3} M $K_4Fe(CN)_6$ were in the blank cell. The solution was clear at 60 min, cloudy at 80 min.

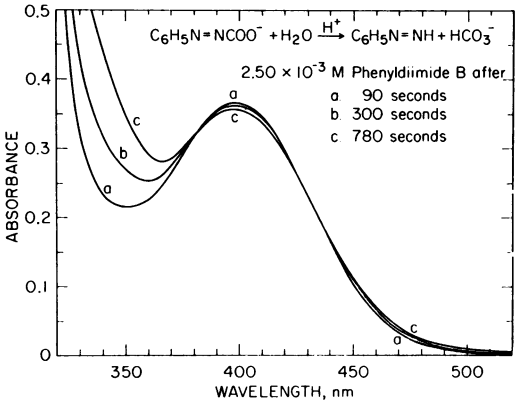


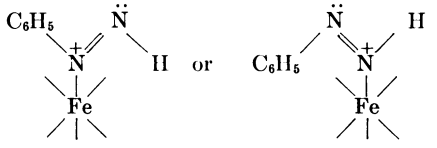
FIG. 6. Visible spectrum of the decarboxylation product of phenylazoformic acid (phenyldiimide B) in phosphate buffer of pH 7.34, μ 0.167. Path length, 1 cm. Times at which absorbance at 400 nm was recorded are shown. Saponified methyl phenylazoformate (0.1077 M in 0.6 NaOH) was added to phosphate buffer to yield 2.50×10^{-3} M $C_6H_5N = NCOO^-$. A volume of 0.6 N HCl equal to the volume of 0.6 N NaOH used was added in order to minimize pH change. The solution became turbid after 1020 sec.

Discussion. The presence of isosbestic points in the spectra of Fig. 1 is consistent with two conclusions. The first is that ferricyanide is completely reduced to ferrocyanide by phenylhydrazine. The second is that the reaction mixture contains only two absorbing species other than ferrocyanide ion. Since phenylhydrazine, present in excess, is one of these, only one product other than ferrocyanide was produced in the oxidation of phenylhydrazine by ferricyanide. Subtraction of the absorption of phenylhydrazine and ferrocyanide from the spectrum of a partially oxidized solution of phenylhydrazine resulted in ultraviolet and visible spectra extremely similar to those of phenyldiimide produced from phenylazoformic acid (Figs. 3–6); consequently the oxidation of 1 mole of phenylhydrazine with 2 moles of potassium ferricyanide near neutral pH is a stoichiometric reaction that results in 1 mole of phenyldiimide and 2 moles of ferrocyanide. This reaction, therefore, can be used to obtain spectral constants of phenyldiimide based upon initial spectra after reacting accurately prepared solutions of highly purified phenylhydrazine hydrochloride and potassium ferricyanide. If the reagents were impure and were not measured accurately, the isosbestic points of Fig. 1 would not have been observed.

The molar extinction coefficients of the product of phenylazoformate decarboxylation was 2–3% lower than those of the product of phenylhydrazine oxida-

tion although the wavelengths of absorption maxima were identical (Table 1). The values obtained from the latter reaction are more reliable because of the greater purity of the starting materials. Methyl phenylazoformate, a high-boiling liquid, was obtained commercially and was not redistilled, and the nitrogen analysis of the sample used was slightly lower than the theoretical value. Purified solutions of phenyldiimide prepared from methyl phenylazoformate in acetonitrile have been prepared by distillation[7]; however, direct and accurate determinations of the concentration of such solutions is difficult. A previous estimate of the extinction coefficient of phenyldiimide in acetonitrile was based on reduction of phenyldiimide to phenylhydrazine,[7] an approach that depends upon estimates of the rate of decomposition of phenyldiimide during reduction and of the efficiency of the reduction itself.

Diimide and aryldiimides bind to heme and heme proteins.[8 10] Phenyldiimide binds to ferrihemoglobin to form a compound with a novel visible absorption spectrum.[8] Phenyldiimide probably is planar and in the *trans* configuration,[7] and its bond with hemoglobin must be with the iron of heme in order to cause the observed change in spectrum.[8] The electronic structures of ferrohemoglobin, ferrihemoglobin, and their respective ligands have been compared by Pauling,[11] who concluded that in ferrihemoglobin compounds, electrons from the ligand, and not the valence electrons of the iron atom, are used to form the bond between ligand molecule and ferrihemoglobin. The structure of the compound between *trans*-phenyldiimide and ferrihemoglobin can be written with the iron atom connected either to the nitrogen atom (N^1) bonded to the phenyl group or the nitrogen atom (N^2) bonded to the hydrogen atom.

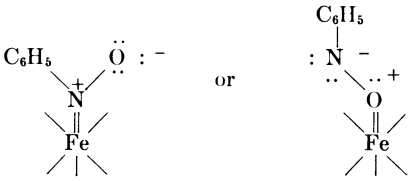


Since both structures are electronically possible, their spatial possibilities were studied with the use of space-filling molecular models[12] of heme and *trans*-phenyldiimide. The unshared electron pair of neither N^1 or N^2 can be brought into contact with the iron of heme at a reasonable bonding angle if *trans*-phenyldiimide is in a planar configuration because of steric hindrance between the phenyl and porphyrin rings. If the ligand is not planar when combined with ferrihemoglobin, and if the phenyl ring is rotated 90° about its bond to N^1, the N^1 of the model of *trans*-phenyldiimide can be connected to the Fe of the model of heme. The plane of the phenyl ring faces the plane of the porphyrin ring at an angle of about 30°. Even after rotation of the phenyl ring, connection between N^2 and Fe of the models is not possible because of steric hindrance. These models suggest, therefore, that the bond between *trans*-phenyldiimide and ferrihemoglobin is formed between N^1 of the former and Fe of the latter.

The mechanism by which phenylhydrazine induces hemolytic anemia has been the subject of much study. Although phenylhydrazine is a reducing agent that reduces ferrihemoglobin to ferrohemoglobin,[13] one of the effects of adding phenylhydrazine to red blood cells is the formation of ferrihemoglobin. Hydrogen

peroxide, which oxidizes ferrohemoglobin to ferrihemoglobin,[14] is formed by the reaction of phenylhydrazine with oxygen or oxyhemoglobin.[15,16] The phenyldiimide formed in the same reaction (Fig. 2) would then combine with ferrihemoglobin to produce phenyldiimideferrihemoglobin. The spectrum of phenyldiimideferrihemoglobin has a weak absorption band that suggests the 630 nm band of acid ferrihemoglobin, but differs in that it is not abolished by the addition of cyanide ion. A component with this property appears in the hemoglobin of phenylhydrazine-treated erythrocytes.[17] The stability of hemoglobin is extremely sensitive to alterations in the vicinity of the hemes.[18,19] Introduction of a large ligand such as phenyldiimide may distort the hemoglobin molecule to an unstable conformation and may be a factor in the formation of Heinz bodies.

Nitrobenzene and aniline also cause hemolytic anemia.[20] Although neither combines with ferrohemoglobin or ferrihemoglobin, their common physiological effect may arise from conversion of both to nitrosobenzene, a compound of intermediate oxidation state, in the erythrocyte. The latter compound reacts with ferrohemoglobin[21,22] and is nearly the same size and shape as phenyldiimide. According to Pauling,[11] ligands of ferrohemoglobin have such a structure as to permit them to combine with the ferroheme group without destroying the electrical neutrality of the iron atoms. Neutrality is maintained by using two electrons of the ligand and two electrons of the iron atom to form a double bond. The structure of nitrosobenzeneferrohemoglobin can be written in accordance with this rule.



Space-filling models of heme and nitrosobenzene can be joined to produce a model of either compound. However, a structure with the formal positive charge on nitrogen would be more stable chemically and is, in fact, supported by kinetic and equilibrium data. Gibson[23] found that the rate of combination of *o*-nitrosotoluene with ferrohemoglobin is about one-fifth that of nitrosobenzene, *m*-nitrosotoluene, or *p*-nitrosotoluene; and Murayama[24] found that the affinity of *o*-nitrosotoluene for hemoglobin is about one-tenth that of nitrosobenzene. Examination of molecular models reveals that a methyl group on the carbon atom *ortho* to the nitrogen atom in nitrosotoluene hinders the joining of the nitrogen to the iron of heme but does not hinder the joining of the oxygen atom to heme. Methyl groups at the *meta* and *para* positions do not hinder the joining of nitrogen to iron. Thus the relative positions of homologous atoms in the postulated structures of phenyldiimideferrihemoglobin and nitrosobenzeneferrohemoglobin are the same. Sulfhemoglobin is the term used to designate compounds of hemoglobin that result from the action of phenylhydrazine and certain drugs.[20,25] These compounds are characterized by inability to transport oxygen and by high absorbance at 620 nm which is unchanged by the addition of cyanide. Both phenyldiimideferrihemoglobin and nitrosobenzeneferrohemo-

globin have these properties. Many of the compounds that induce Heinz body formation in erythrocytes deficient in glucose-6-phosphate dehydrogenase (EC 1.1.1.49)[26] are aromatic amines and amides. It has been shown that both the oxidation of aniline to nitrosobenzene[27] and the reduction of nitrosobenzene to aniline[28] occur in blood. These facts suggest the possibility that Heinz bodies are formed in such eythrocytes from hemoglobin molecules destabilized by the binding of large arylnitroso compounds, which accumulate in enzyme-deficient cells because of inability of these cells to reverse the oxidation of arylamines.

Abbreviations used: C_f, molar concentration of ferrocyanide; C_p, molar concentration of phenylhydrazine.

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