The Oxidation of Phenylhydrazine: Superoxide and Mechanism[†]

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ABSTRACT: The oxidation of phenylhydrazine in buffered aqueous solutions is a complex process involving several intermediates. It can be initiated by metal cations, such as Cu^{2+}; in which case EDTA acts as an inhibitor. It can also be initiated by oxyhemoglobin; in which case chelating agents do not interfere. Superoxide radical is both a product of this reaction and a chain propagator. The formation of O_2^- could be demonstrated in terms of a reduction of nitroblue tetrazolium, which was prevented by superoxide dismutase. The importance of O_2^- in carrying the reaction chains was shown by the inhibition of phenylhydrazine oxidation by superoxide dismutase. Hydrogen peroxide accumulated during the reaction and could be detected with catalase. The progress of this oxidation could be monitored in terms of oxygen consumption and by following increases in absorbance at 280 or 320 nm. The oxidation was markedly autocatalytic and superoxide dismutase had the effect of extending the lag period. The absorbance at 280 nm was due to an intermediate which first accumulated and was then consumed. This intermediate appears to be benzenediazonium ion. The absorbance at 320 nm was due to a stable product, which was not identified. The time course of oxygen consumption paralleled the increase in absorbance at 320 nm and lagged behind the changes at 280 nm. Exogenous benzenediazonium ion accelerated the oxidation of phenylhydrazine and eliminated the lag phase. Benzenediazonium ion must therefore react with phenylhydrazine to produce a very reactive intermediate, possibly phenyldiazene. A mechanism was proposed which is consistent with the data. The intermediates and products of the oxidation of phenylhydrazine include superoxide radical, hydrogen peroxide, phenylhydrazyl radical, phenyldiazene, and benzenediazonium ion. This is a minimal list: others remain to be detected and identified. It appears likely that the diverse biological effects of phenylhydrazine are largely due to the reactivities of these intermediates and products.

Phenylhydrazine has long been known as a hemolytic agent (Warburg et al., 1931; Lemberg and Legge, 1942; Beaven and White, 1954) and has been shown to increase the production of H_2O_2 within intact human erythrocytes (Cohen and Hochstein, 1964). Several of the effects of phenylhydrazine are oxygen-dependent. These include its inactivation of papain (Allison and Swain, 1973), amine oxidase (Patek and Hellerman, 1974), lactoperoxidase and triosephosphate dehydrogenase (Allison et al., 1973), horse radish peroxidase and thyroid peroxidase (Hidaka and Udenfriend, 1970; Hidaka et al., 1970), its inhibition of the respiration of mitochondria and of submitochondrial particles (Asami, 1968), and its desensitization of grasshopper muscle (McDonald, 1972). Aside from the involvement of phenyldiazene as an intermediate (Cauquis and Genies, 1968; Itano, 1970), the catalytic effect of Cu^{2+} (Eberson and Persson, 1962; Audrieth and Ogg, 1951) and the formation of H_2O_2 (Cohen and Hochstein, 1964; Audrieth and Ogg, 1951), very little is known about the air oxidation of phenylhydrazine in buffered aqueous solutions.

The reduction of molecular oxygen to H_2O_2 frequently occurs in univalent steps with O_2^- as an intermediate. This has been shown to be the case during the reduction of oxygen by epinephrine (Misra and Fridovich, 1972b), pyrogallol (Marklund and Marklund, 1974), oxyhemoglobin (Misra and Fridovich, 1972c), reduced ferredoxins (Misra and Fridovich, 1971) and leucoflavins, hydroquinones, and thiols (Misra and Fridovich, 1972a; Misra, 1974). It therefore seemed reasonable that this might also be true of the oxidation of phenylhydrazine; in which case O_2^- and other radical intermediates would have to be considered as possible agents of the biological effects of this compound. The ability of O_2^- to reduce nitroblue tetrazolium (Beauchamp and Fridovich, 1971) and the availability of superoxide dismutases, which catalytically scavenge O_2^- (Fridovich, 1974), provided the means for probing the oxidation of phenylhydrazine. The report which follows describes studies of the mechanism of oxidation of phenylhydrazine. O_2^-, phenyldiazene, and benzenediazonium ion appear to be intermediates and a tentative mechanism is proposed. While this work was in progress, O_2^- was reported to be a product of the oxidation of phenylhydrazine by oxyhemoglobin (Goldberg and Stern, 1975).

Materials and Methods

Hemoglobin. Heparinized human blood was centrifuged and the erythrocytes were washed three times with isotonic saline and were then lysed by admixture of an equal volume of water. Cell stroma were removed by centrifugation at 100 000*g* for 1 h. A small amount of $Na_2S_2O_4$ (~1.0 mg/ml) was added to ensure complete reduction of the hemoglobin, which was then separated from smaller molecules by gel exclusion chromatography of 12 ml of the lysate on a 4 × 80 cm column of Sephadex G-75, which had been equilibrated and was eluted with nitrogen-purged 0.05 M potassium phosphate-0.10 M KCl at pH 7.4. The hemoglobin-containing eluate was found to be free of superoxide dismutase. It was converted to oxyhemoglobin by oxygenation and to methemoglobin by treatment with potassium ferricyanide

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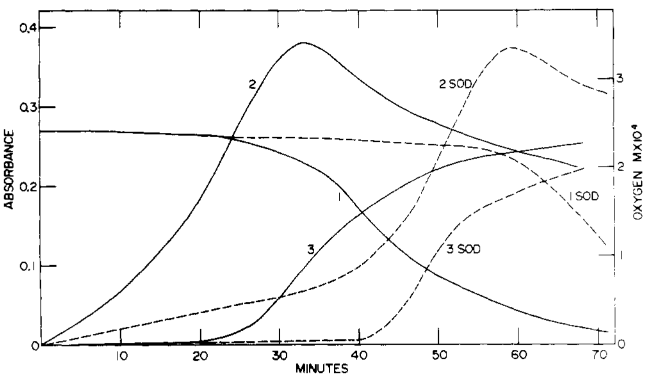

FIGURE 1: The effects of superoxide dismutase on the kinetics of oxidation of phenylhydrazine. Reaction mixtures contained 0.2 mM phenylhydrazine in 50 mM sodium carbonate at pH 10.2 and at 25 °C. SOD denotes the presence of 3 μg/ml of superoxide dismutase. Thus lines 1 and 1 SOD show oxygen uptake in the absence and in the presence of superoxide dismutase while lines 2 and 2 SOD similarly depict changes in absorbance at 280 nm and lines 3 and 3 SOD illustrate changes at 320 nm.

followed by extensive dialysis. All of these manipulations were carried out at 0–4°C. The spectral properties of the oxyhemoglobin and methemoglobin were in accord with the literature (Wright, 1963; Salvati et al., 1969). Concentrations of oxy- and of methemoglobin were estimated on the basis of molar extinctions of $15.3 \times 10^3\ M^{-1}\ cm^{-1}$ at 540 nm and of $9.5 \times 10^3\ M^{-1}\ cm^{-1}$ at 500 nm, respectively (Lemberg and Legge, 1949).

Other Substances. Superoxide dismutase was isolated from bovine blood and was assayed as previously described (McCord and Fridovich, 1969). Bovine liver catalase was obtained from the Sigma Chemical Company and was freed of contaminating superoxide dismutase by gel exclusion chromatography on Sephadex G-75. Phenylhydrazine hydrochloride was from Eastman Chemicals and was recrytallized from ethanol and subsequently stored at 0–4 °C in the dark. *N*-1-Naphthylethylenediamine dihydrochloride was also from Eastman.

Assays. Spectrophotometric assays were conducted at 25 °C with a Gilford Model 2000 or a Cary Model 15. Reactions under controlled atmospheres were conducted in cuvettes which allowed purging the reaction volume with the desired gas (Lazarow and Cooperstein, 1954). Oxygen uptake was followed with a Clark electrode assembly on a Gilson Medical electronic oxygraph. Reduction of nitroblue tetrazolium to the corresponding formazan was followed at 560 nm in the absence of hemoglobin, but at 590 nm in its presence. The latter wavelength is one at which oxy- and methemoglobins are isosbestic. The oxidation of oxy- to methemoglobin was followed at 540 nm using an extinction coefficient of $9.2 \times 10^3\ M^{-1}\ cm^{-1}$ (Lemberg and Legge, 1949). Catalase was assayed by the method of Beers and Sizer (1952) modified as previously described (Misra and Fridovich, 1971). Oxidations of phenylhydrazine were initiated by diluting a cold concentrated water solution of the hydrochloride into the desired buffer at 25 °C. Stock solutions of 10 mM phenylhydrazine hydrochloride were prepared fresh daily and kept on ice.

Results

Catalysis by Metals. Although stable in acid solutions, phenylhydrazine oxidized in neutral or alkaline buffers. This autoxidation was catalyzed by traces of metals. Thus when the oxidation was followed in terms of increased absorbance at 280 nm, EDTA inhibited and Cu^{2+} accelerated. At pH 10.2, in 0.05 M carbonate buffer, 5 μM or 10 μM EDTA inhibited 37 or 80%, respectively. Greater levels of EDTA, up to 100 μM, did not cause further inhibition. $CuCl_2$, at 2 μM, caused a 2.7-fold augmentation of the rate. The reaction could also be accelerated by raising the concentration of oxygen. Thus the rate was 2.6 times faster under 100% oxygen than under air.

Kinetics. The oxidation of phenylhydrazine was markedly autocatalytic and involved at least one intermediate and one product, which absorbed in the ultraviolet. When oxygen uptake was monitored, there was a long lag followed by a rapidly accelerating oxygen consumption, which then gradually approached a limit. Absorbance at 320 nm also showed a long lag followed by an autocatalytic increase to a plateau. In contrast, absorbance at 280 nm began to increase immediately, albeit with evidence of autocatalysis, to a peak, which was followed by a decline. All of this is shown in Figure 1, which also shows that superoxide dismutase markedly inhibited the reaction. The major effect of superoxide dismutase was a prolongation of the lag phase of this oxidation. Although not shown in Figure 1, this effect of superoxide dismutase was related to its concentration and the lags could be lengthened by raising the concentration of this enzyme. Thus the time required for an absorbance increase of 0.150 at 320 nm, under the conditions of Figure 1, was 38 min in the absence of superoxide dismutase, 42 min in the presence of 0.33 μg/ml, 52 min with 3.3 μg/ml, 88 min with 16 μg/ml, and 102 min with 33 μg/ml of this enzyme. It was striking that the maximum rate of oxygen uptake, or of absorbance increase at 280 nm or at 320 nm, was hardly affected by superoxide dismutase, whose major effect was thus a pronounced prolongation of the lag. H_2O_2 was a product of oxygen reduction during this oxidation. Thus addition of catalase, at any phase of the reaction, caused a sudden evolution of oxygen. The amount of oxygen evolved by catalase was always less than half of that consumed up to the moment of addition of catalase and the fraction of the oxygen consumed, which could be accounted for as H_2O_2, declined during the course of the reaction. H_2O_2 was therefore either not a stoichiometric product of oxygen reduction at all stages of this oxidation or alternately it was not fully stable in these reaction mixtures.

These results demonstrate that the 280-nm absorption was due to one or more intermediates, since it first increased and then declined. The 320-nm absorbing species appears to be a stable product, since it accumulated to a plateau. The 280-nm species was somehow a precursor of that absorbing at 320 nm. This is made clearer by Figure 2, which shows changes in the ultraviolet during the oxidation of phenylhydrazine. It is evident that the absorption at 280 nm appeared before there was any change at 320 nm. It is also apparent that the absorption maximum, which first appeared at 280 nm, intensified and shifted slightly to shorter wavelengths, during the first half hour of the reaction, and then diminished in intensity, while shifting once again to longer wavelengths. In contrast, absorption at 320 nm increased to a stable plateau after a prolonged lag.

The lengthening of the lag period by superoxide dismutase indicates that O_2^- was produced at an early stage in the reaction and that it was important for propagating the reaction chains. However, since superoxide dismutase did not affect the maximum slopes of the lines in Figure 1, we must conclude that O_2^- was of importance only during the lag phase of the reaction. It could, in fact, be shown that

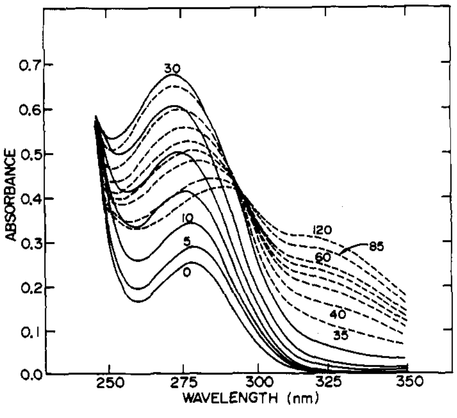

FIGURE 2: Changes in ultraviolet absorption during the oxidation of phenylhydrazine. The reaction mixture contained 0.2 mM phenylhydrazine in 50 mM sodium carbonate at pH 10.2 and at 25 °C. Absorption spectra were recorded at intervals against a buffer blank. The spectra depicted with solid lines were recorded at 5-min intervals starting at time = 0 and reaching maximum absorbance at 270 nm at 30 min. The broken lines were recorded at 35, 40, 45, 50, 55, 60, 85, and 120 min, respectively. Some of these spectra have numbers showing the minutes elapsed at the time the spectrum was recorded.

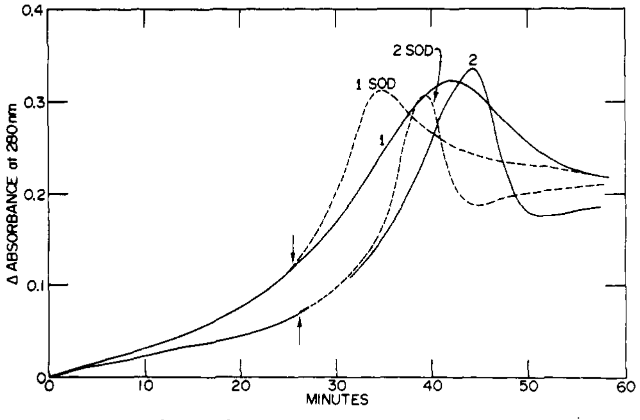

FIGURE 3: The effects of superoxide dismutase added late in the reaction. Reaction mixtures contained 0.16 mM phenylhydrazine and 50 mM sodium carbonate at pH 10.2 and 25 °C, in the absence (1 and 1 SOD) and in the presence (2 and 2 SOD) of 500 units of catalase. At the arrows 64 μg/ml of superoxide dismutase was added to identical reaction mixtures and gave lines 1 SOD and 2 SOD. The unit of catalase was that previously defined (Misra and Fridovich, 1971).

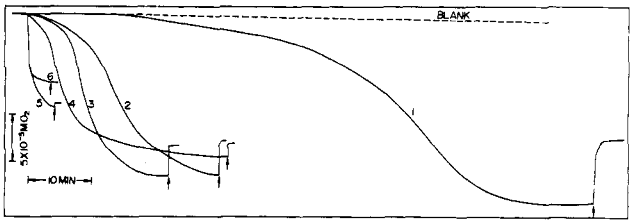

FIGURE 4: Effects of ferricyanide. Reaction mixtures contained 0.1 mM phenylhydrazine in 50 mM sodium carbonate at pH 10.2 and 25 °C. Additional components were: (1) none; (2) 0.05 mM ferricyanide plus 15 μg/ml of superoxide dismutase; (3) 0.05 mM ferricyanide; (4) 0.1 mM ferricyanide; (5) 0.15 mM ferricyanide; and (6) 0.2 mM ferricyanide. At the arrows 500 units of catalase was added. The total reaction volume was 2.5 ml.

O_2^- actually retarded the oxidation of phenylhydrazine once the lag phase was over. This is shown in Figure 3. In this case superoxide dismutase was added 27.5 min after the reaction was started and it was seen to accelerate the reaction. Thus superoxide dismutase present at the outset, as in Figure 1, inhibited the reaction; whereas superoxide dismutase added after the lag was over, actually accelerated the oxidation. Catalase, present from the outset, slightly inhibited the oxidation of phenylhydrazine but did not modify the effect of superoxide dismutase. Once the lag was over, it thus appears that chain propagation was primarily due to species other than O_2^- and further that O_2^- could then scavenge these other species and thus inhibit.

Since phenyldiazene is a likely intermediate in the oxidation of phenylhydrazine (Cauquis and Genies, 1968; Itano, 1970) and since ferricyanide is known to rapidly oxidize phenylhydrazine to the diazene (Huang and Kosower, 1968a; Itano, 1970), the effect of ferricyanide on the oxidation of phenylhydrazine was explored. As shown in Figure 4, potassium ferricyanide eliminated the lag in the oxidation of phenylhydrazine and induced a very rapid consumption of oxygen. The data in Figure 4 were obtained by adding phenylhydrazine to aerobic reaction mixtures containing ferricyanide. When phenylhydrazine was mixed with ferricyanide under anaerobic conditions and this mixture then rapidly added to the aerobic buffer, under the Clarke electrode, similar results were obtained. This indicates that a product of the reaction of ferricyanide with phenylhydrazine, which was relatively stable in the absence of oxygen, was responsible for the rapid O_2 uptake seen in Figure 4. This product is presumed to have been phenyldiazene. The failure to accumulate H_2O_2, when ferricyanide was added, was due to its not being produced rather than to its secondary elimination by reaction with ferricyanide. Ferricyanide did oxidize H_2O_2 but ferrocyanide had no effect upon it and the anaerobic admixture of phenylhydrazine with ferricyanide gave ferrocyanide and phenyldiazene and this mixture then consumed O_2 and produced no H_2O_2. These results were not surprising in view of statements in the literature (Huang and Kosower, 1968b; Tsuji and Kosower, 1971) that phenyldiazene, although reasonably stable in the absence of oxygen, reacts very rapidly with oxygen. The great rate at which phenyldiazene reacts with oxygen precludes the possibility that it could accumulate significantly during the reaction. The 280-nm absorbing intermediate could not therefore be phenyldiazene. Furthermore phenyldiazene has been reported to absorb maximally at 269.5 nm (Huang and Kosower, 1968a; Itano, 1970). The total oxygen consumption, shown in Figure 4, declined as the amount of ferricyanide added was increased. This effect reached its limit when 2 mol of ferricyanide had been added per mol of phenylhydrazine. Superoxide dismutase, present at the start of the reaction, inhibited weakly in the presence of one ferricyanide per phenylhydrazine and not at all in the presence of two ferricyanides per phenylhydrazine. It must be concluded that the oxidation of phenylhydrazine can be accelerated by phenyldiazene and that the oxidation of phenyldiazene is very rapid and does not produce H_2O_2 and does not involve O_2^- as a chain propagator.

The Reduction of Nitroblue Tetrazolium. O_2^- can reduce nitroblue tetrazolium to the corresponding formazan (Beauchamp and Fridovich, 1971). If O_2^- is an intermediate in the oxidation of phenylhydrazine it should be able to reduce the tetrazolium and superoxide dismutase should inhibit this reduction. Figure 5 demonstrates that superoxide dismutase did powerfully inhibit the reduction of the tetrazolium which accompanied the oxidation of phenylhydrazine. Thus as little as 0.15 μg/ml of superoxide dismutase caused 50% inhibition. This verifies that the autoxidation of phenylhydrazine does produce O_2^-. Superoxide dismutase

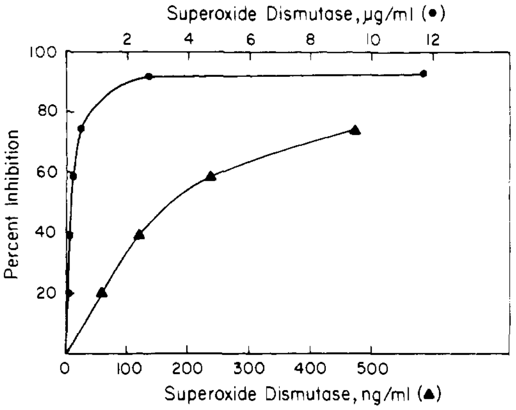

FIGURE 5: The effect of superoxide dismutase on the reduction of nitroblue tetrazolium by phenylhydrazine. Reaction mixtures contained 0.2 mM nitroblue tetrazolium, 4.5 mM phenylhydrzazine, 50 mM sodium carbonate at pH 10.2 and 25 °C, and the indicated concentrations of superoxide dismutase.

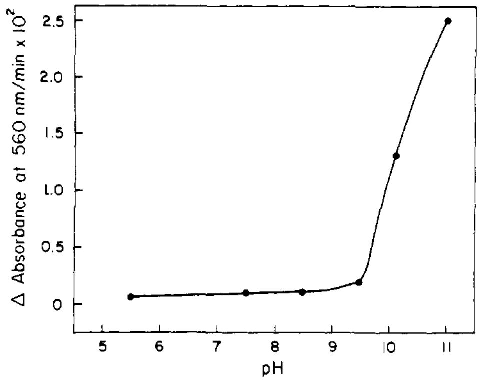

FIGURE 6: The effect of pH on the reduction of nitroblue tetrazolium by phenylhydrazine. Reaction mixtures contained 2.5 mM phenylhydrazine and 0.25 mM nitroblue tetrazolium in the following aerobic buffers: pH 5.5, 0.1 M sodium acetate; pH 7.8, 0.05 M potassium phosphate; pH 9.5, 0.1 M Tris-Cl; pH 10.2, 0.05 M sodium carbonate; and pH 11.0, 0.1 M glycine.

was previously seen to inhibit the oxidation of phenylhydrazine, by prolonging the lag phase, thus exposing the importance of O_2^- as a chain carrier, early in the reaction. Since nitroblue tetrazolium also scavenges O_2^-, albeit not catalytically, the tetrazolium should also inhibit the oxidation of phenylhydrazine. It did have this effect and the high concentration of phenylhydrazine, used to get the data in Figure 5, was needed to counteract this inhibitory effect of the tetrazolium.

Phenylhydrazine is a good reductant and might be expected to directly reduce nitroblue tetrazolium, without the mediation of oxygen. This was the case. Thus, under anaerobic conditions, 3.2 mM phenylhydrazine in 50 mM carbonate at pH 10.2 and 25 °C acted upon 0.25 mM tetrazolium to cause an increase in absorbance at 560 nm of 0.055/min and 12 μg/ml of superoxide dismutase had no effect on this anaerobic reaction. In contrast, when the same reaction was carried out aerobically, 2 μg/ml of superoxide dismutase caused 90% inhibition of the rate of reduction of the tetrazolium. Thus, in the absence of oxygen, phenylhydrazine directly reduced the tetrazolium and superoxide dismutase had no effect; whereas in the presence of oxygen the phenylhydrazine reduced oxygen to O_2^-, which then reduced the tetrazolium and superoxide dismutase could intercept O_2^- and thus inhibit. We must also conclude that phenylhydrazine reacts much more rapidly with oxygen than with the tetrazolium because the anaerobic rate of formazan production vastly exceeded that seen aerobically in the presence of superoxide dismutase. The aerobic reduction of nitroblue tetrazolium by phenylhydrazine was very much a function of pH. This is shown in Figure 6. Superoxide dismutase inhibited this tetrazolium reduction over this entire range of pH.

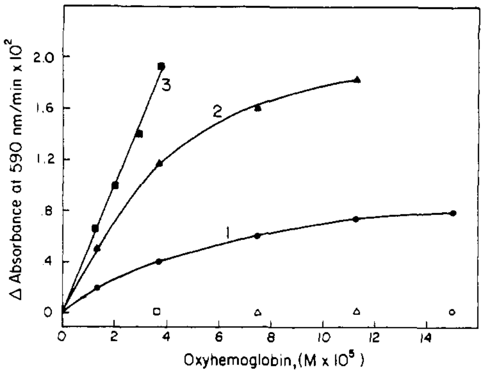

FIGURE 7: The production of O_2^- by phenylhydrazine plus oxyhemoglobin. Reaction mixtures contained 0.25 mM nitroblue tetrazolium and the indicated concentrations of oxyhemoglobin in 50 mM potassium phosphate at pH 7.4 and 25 °C. The concentrations of phenylhydrazine were: line 1 = 0.08 mM; line 2 = 0.2 mM; and line 3 = 0.4 mM. The open points on the abscissa show the complete inhibition which was caused by 50 μg/ml of superoxide dismutase. The open points were obtained at the phenylhydrazine concentrations used to get the corresponding filled points.

Oxidation of Phenylhydrazine by Oxyhemoglobin. Nitroblue tetrazolium was not measurably reduced to the formazan by either 0.06 mM oxyhemoglobin or by 0.2 mM phenylhydrazine, acting separately in 50 mM potassium phosphate at pH 7.4 and 25 °C. It was, however, rapidly reduced by the combination of these agents and this reduction was entirely inhibited by 50 μg/ml of superoxide dismutase. Figure 7 presents the rate of reduction of nitroblue tetrazolium as a function of [oxyhemoglobin] and at several concentrations of phenylhydrazine. The open points near the abscissa illustrate the total inhibition caused by superoxide dismutase. It follows that the reaction of phenylhydrazine with oxyhemoglobin generates O_2^-. Methemoglobin was unable to replace oxyhemoglobin in this reaction with phenylhydrazine and EDTA at 1.0 mM had no effect upon the rate of reduction of the tetrazolium.

Oxyhemoglobin is partially a superoxoferriheme, due to reversible transfer of an electron from the heme iron to the bound oxygen (Wittenberg et al., 1970; Yamamoto et al., 1973). It appears likely that this bound oxygen is a more potent oxidant than free oxygen and that it can directly react with phenylhydrazines as follows:

$$\mathrm{HbFe^{3+}{-}O_2^- + Ph{-}NH{-}NH_2 \xrightarrow{+H^+} HbFe^{3+} + H_2O_2 + Ph{-}NH{-}NH\cdot} \quad (1)$$

The phenylhydrazyl radical, so generated, could then react with oxygen, to generate the O_2^-, which was responsible for the reduction of nitroblue tetrazolium. Thus:

$$\mathrm{Ph{-}NH{-}NH\cdot + O_2 \rightarrow Ph{-}N{=}NH + H^+ + O_2^-} \quad (2)$$

In this way oxyhemoglobin could initiate an oxidation of phenylhydrazine which was insensitive to EDTA because trace metals played no part in the initiation process.

Detection of Diazobenzene. Both phenyldiazene and ben-

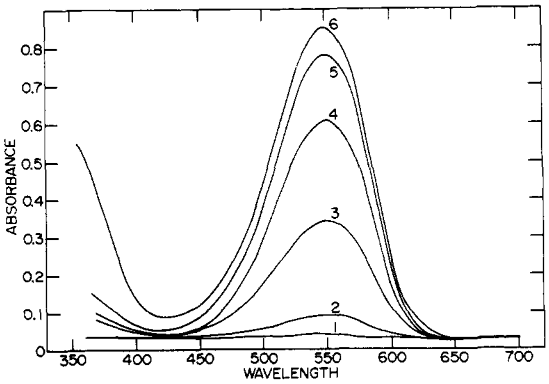

FIGURE 8: The coupling of N-1-naphthylethylenediamine with benzenediazonium ion and with an intermediate of the oxidation of phenylhydrazine. Reaction mixtures containing 0.2 mM phenylhydrazine in 50 mM sodium carbonate, in a total volume of 2.4 ml, at pH 10.2 and 25 °C were incubated for variable times and were then treated with 0.1 ml of 0.5% N-1-naphthylethylenediamine in 3.0 N HCl and after 2 additional hours the absorption spectra were recorded. The times which elapsed between initiation of the oxidation of phenylhydrazine and the addition of the coupling agent were: line 1, 0.0 min; line 2, 5 min; line 3, 10 min; line 4, 15 min; and line 5, 20 min. Line 6 was obtained by substituting 2.4 ml of 0.02 mM benzenediazonium ion for the phenylhydrazine.

zenediazonium ion are reasonable intermediates of the oxidation of phenylhydrazine. Indeed, phenylhydrazine is easily prepared by the reduction of benzenediazonium ion (Millar and Springall, 1969). Diazonium compounds readily couple with N-1-naphthylethylenediamine and yield colored products (Bratton and Marshall, 1939). Benzenediazonium ion, prepared by diazotization of aniline with nitrous acid, gave a red product upon coupling with this reagent. Aliquots removed during the oxidation of phenylhydrazine and treated with N-1-naphthylethylenediamine gave an identical color. This is shown in Figure 8. It is apparent that the oxidation of phenylhydrazine causes the accumulation of an intermediate, which couples with N-1-naphthylethylenediamine and in so doing yields a product whose spectrum is identical with that given by coupling benzenediazonium ion with this naphthyl reagent. Furthermore, the rate of color development, during the reaction of authentic benzenediazonium ion with N-1-naphthylethylenediamine, was comparable to that seen with air-oxidized phenylhydrazine.

Benzenediazonium ion absorbs maximally at 280 nm at pH 11 (Porai-Koshits, 1960). If the 280-nm absorbing compound, which transiently accumulated during the oxidation of phenylhydrazine, is benzenediazonium ion, then the yield of red product, upon coupling with the naphthalene reagent, should follow the same time course as the absorbance at 280 nm. Figure 9 demonstrates that this was the case. It appears likely, therefore, that benzenediazonium ion is an intermediate in the oxidation of phenylhydrazine.

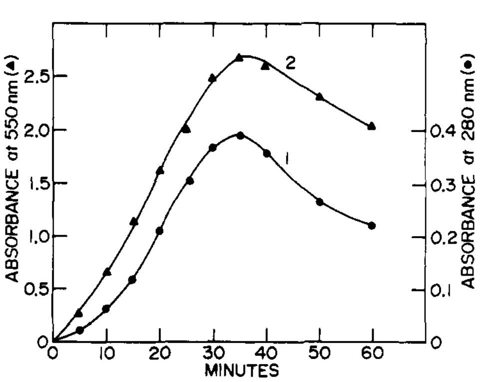

FIGURE 9: Kinetics of benzenediazonium ion production. Phenylhydrazine (0.2 mM) was allowed to oxidize in 50 mM sodium carbonate at pH 10.2 and 25 °C. Changes in absorbance at 280 nm were recorded and are shown on line 1. At the same time 2.4-ml aliquots of the reaction mixture were treated with 0.1 ml of 0.5% N-1-naphthylethylenediamine in 3.0 N HCl and the absorbances were read at 550 nm, after overnight incubation in the dark. These results are shown on line 2.

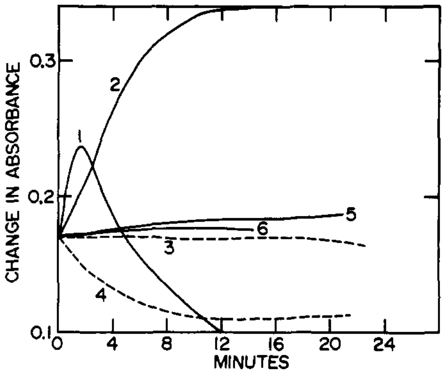

FIGURE 10: The effect of benzenediazonium ion on phenylhydrazine—spectrophotometric monitoring. Benzenediazonium ion (0.1 mM) was mixed with 0.2 mM phenylhydrazine in 50 mM sodium carbonate at pH 10.2 and 25 °C. Lines 1 and 2 present the changes in absorbance at 280 and at 320 nm, respectively, which were seen aerobically. Lines 3 and 4 show the corresponding changes seen anaerobically while lines 5 and 6 show the changes at these wavelengths when only benzenediazonium ion was present under aerobic conditions.

Catalysis by Benzenediazonium Ion. It appeared possible that the stable 320-nm absorbing product of the oxidation of phenylhydrazine might be formed through the coupling of benzenediazonium ion with another, as yet unidentified, intermediate. It was also considered that benzenediazonium ion might have a catalytic effect and thus account for the autocatalysis seen late in the oxidation of phenylhydrazine, even in the presence of superoxide dismutase. To explore these possibilities benzenediazonium ion was added to phenylhydrazine and its effects were noted. Figure 10 illustrates that benzenediazonium ion markedly accelerated the oxidation of phenylhydrazine, as judged by increases in absorbance at 280 and 320 nm. Indeed, it eliminated the lags which are shown in Figure 1 and which characterized these changes in its absence and it made the absorbance increase at 280 nm an even more evanescent phenomenon. These changes were dependent upon the presence of oxygen so they do not represent any anaerobic coupling of benzenediazonium ion with phenylhydrazine itself. Under anaerobic conditions there was no change in absorbance at 320 nm. Benzenediazonium ion itself did not react significantly with oxygen since, in the absence of phenylhydrazine it showed only slight increases in absorbance at 280 and 320 nm. The effects of benzenediazonium ion on phenylhydrazine were also monitored in terms of oxygen consumption. These results are shown in Figure 11. When benzenediazonium ion was added to the carbonate buffer in the absence of phenylhydrazine it caused a small and transient oxygen consumption. The data in Figure 11 were corrected for this effect by incubating the benzenediazonium ion in the buffer for several minutes, until this oxygen uptake ceased, prior to the addition of phenylhydrazine and activation of the recorder. It is apparent that benzenediazonium ion strongly catalyzes the oxidation of phenylhydrazine.

The effects of benzenediazonium ion could be explained

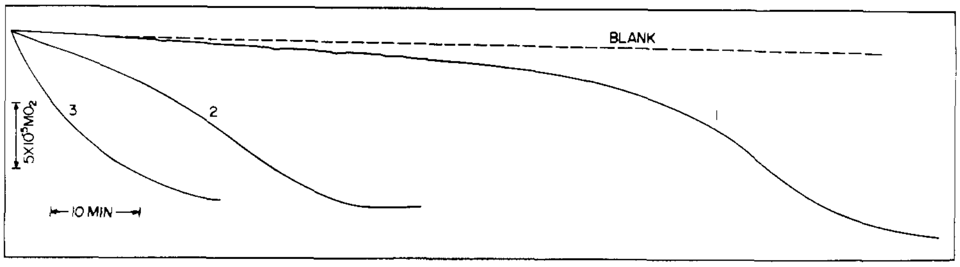

FIGURE 11: The effect of benzenediazonium ion on phenylhydrazine—oxygen consumption. Reaction mixtures contained 0.12 mM phenylhydrazine in 50 mM sodium carbonate at pH 10.2 and 25°. Additional components were: line 1, none; line 2, 0.05 mM benzenediazonium ion; and line 3, 0.1 mM benzenediazonium ion. These levels of the diazo compound, in the absence of phenylhydrazine, did not cause significant oxygen uptake under these conditions.

by the following reactions:

$$\mathrm{Ph{-}N{\equiv}N^+ + Ph{-}NH{-}NH_2 \rightarrow 2Ph{-}N{=}NH + H^+} \tag{3}$$

$$\mathrm{Ph{-}N{=}NH + \tfrac{1}{2}O_2 + H^+ \rightarrow Ph{-}N{\equiv}N^+ + H_2O} \tag{4}$$

Thus benzenediazonium ion might catalyze the oxidation of phenylhydrazine by converting it to phenyldiazene; which is known to react very rapidly with oxygen (Huang and Kosower, 1968b; Tsuji and Kosower, 1971). Since the oxidation of phenyldiazene yields benzenediazonium ion, the effect of benzenediazonium ion would be catalytic and its production during the oxidation of phenylhydrazine would lend a strong autocatalytic aspect to the reaction. It is therefore apparent why exogenous benzenediazonium ion would eliminate the lag in the oxidation of phenylhydrazine. Benzenediazonium ion readily couples with electron-rich aromatic rings and it is possible that it couples with some other intermediate to yield the 320-nm absorbing product. Since benzenediazonium ion did not accelerate the appearance of 320-nm absorbance from phenylhydrazine, unless oxygen was present, it could not have generated this product by reaction with phenylhydrazine, per se. An attempt can be made to explain the absorbance changes seen in Figure 10, in the absence of oxygen. Thus benzenediazonium ion has an absorption maximum at 280 nm (Porai-Koshits, 1960) while phenyldiazene has one at 269.5 nm (Huang and Kosower, 1968a; Itano, 1970). Phenyldiazene thus absorbs less than does benzenediazonium ion at 280 nm; but each benzenediazonium ion which reacted with phenylhydrazine produced two molecules of phenyldiazine. This compensated for their different absorptions at 280 nm and no significant change was seen. At 320 nm, however, the absorption by benzenediazonium ion so exceeds that of phenyldiazene that the phenyldiazene produced could not compensate for the benzenediazonium ion consumed and the absorbance at 320 nm decreased anaerobically (Figure 10). It is also possible to try to explain the very rapid increase and subsequent decrease in absorbance at 280 nm seen aerobically in Figure 10. Thus exogenous benzenediazonium ion not only accelerated the rate of production of more benzenediazonium ion by reactions 3 plus 4 but also accelerated the rate of consumption of benzenediazonium ion by unspecified processes, which could include condensation with phenylhydrazine to give a tetrazene which might then decompose (Clusius and Craubner, 1955; Smith, 1966). Thus in the presence of exogenous benzenediazonium ion more of it was rapidly generated at the outset and absorbance at 280 nm rose; but then the processes which consumed it began to predominate and absorbance at 280 nm fell sharply (Figure 10).

Discussion

The observations reported above can be discussed and explained most conveniently by proposing sequences of reactions. Thus the long lag phase, the lengthening of this lag by superoxide dismutase and the detection of O_2^- production with nitroblue tetrazolium suggest a free radical chain reaction in which O_2^- is a chain propagator. An initiation step involving a metal cation will account for catalysis by Cu^{2+} and inhibition by EDTA, hence the following reactions:

$$\mathrm{Ph{-}NH{-}NH_2 + Me^{2+} \rightarrow Me^+ + H^+ + Ph{-}NH{-}NH\cdot} \tag{a}$$

$$\mathrm{Ph{-}NH{-}NH\cdot + O_2 \rightarrow H^+ + O_2^- + Ph{-}N{=}NH} \tag{b}$$

$$\mathrm{O_2^- + H^+ + Ph{-}NH{-}NH_2 \rightarrow H_2O_2 + Ph{-}NH{-}NH\cdot} \tag{c}$$

$$\mathrm{O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2} \tag{d}$$

$$\mathrm{2Ph{-}NH{-}NH\cdot \rightarrow Ph{-}N{=}NH + Ph{-}NH{-}NH_2} \tag{e}$$

Reaction (a) provides for initiation, reactions (b) and (c) for chain propagation, and reactions (d) and (e) for chain termination. Termination reaction (d) is catalyzed by superoxide dismutase and accounts for the prolongation of the lag by this enzyme.

Phenylhydrazine is stable in acid solution presumably because its protonated form does not react with the metal cation as in (a). The pK_a of phenylhydrazine is 5.1 (Millar and Springall, 1969). This could not account for the great increase in the rate of O_2^- production seen above pH 9.5 in Figure 5. We could explain this by supposing an ionization of the phenylhydrazyl radical and a much more rapid reaction of the radical anion, so generated, with oxygen, as follows:

$$\mathrm{Ph{-}NH{-}NH\cdot \rightarrow H^+ + Ph{-}NH{-}N\cdot^-} \tag{f}$$

$$\mathrm{Ph{-}NH{-}N\cdot^- + O_2 \rightarrow Ph{-}N{=}NH + O_2^-} \tag{g}$$

Ferricyanide, which is known to rapidly oxidize phenylhydrazine to the diazene, eliminated the lag, accelerated the oxidation of phenylhydrazine, and also eliminated production of H_2O_2. Furthermore, benzenediazonium ion, which was demonstrably an intermediate, also accelerated the oxidation and superoxide dismutase, which inhibited so effectively when present at the outset, actually augmented the reaction, when added after the lag was partially over. The following reactions are offered in partial explanation of these effects:

$$\mathrm{Ph{-}NH{=}NH + \tfrac{1}{2}O_2 \xrightarrow{\text{very}} \xrightarrow{\text{fast}} H_2O + Ph{-}N{\equiv}N^+} \tag{h}$$

$$\mathrm{Ph{-}N{\equiv}N^+ + Ph{-}NH{-}NH_2 \rightarrow 2Ph{-}N{=}NH + H^+} \qquad \text{(i)}$$

$$\mathrm{Ph{-}N{\equiv}N^+ + O_2^- \rightarrow Ph{-}N{=}N\cdot + O_2} \qquad \text{(j)}$$

$$\mathrm{Ph{-}N{=}N\cdot + O_2^- + H^+ \rightarrow Ph{-}N{=}NH + O_2} \qquad \text{(k)}$$

Here reaction (h) provides for the rapid oxygen consumption caused by oxidizing the phenylhydrazine to the diazene with ferricyanide; reaction (i) accounts for the catalytic effect of benzenediazonium ion and (h) and (i) provide for autocatalysis independent of O_2^-. In fact a reaction of O_2^- with benzenediazonium ion, as proposed by reactions (j) and (k) would explain why superoxide dismutase actually accelerated the latter part of the overall reaction.

We are now forced to think of the biological effects of phenylhydrazine in a new light. Thus oxyhemoglobin not only produces H_2O_2 and methemoglobin in its reaction with phenylhydrazine but also yields the phenylhydrazyl radical which in turn can generate O_2^-, phenyldiazene, benzenediazonium ion, and possibly other, as yet undetected, intermediates. Because of the speed with which it reacts with oxygen, we cannot expect phenyldiazene to accumulate to any degree during the oxidation of phenylhydrazine. The situation is quite different for benzenediazonium ion; since it does accumulate substantially, during this oxidation. It is now tempting to attribute some of the biological effects of phenylhydrazine to the intermediates produced during its oxidation.

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