The Oxygen Effect on Deoxyribonucleic Acid Inactivation by Hydroxylamines*

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ABSTRACT: Hydroxylamine (HA) and some of its derivatives containing free NOH groups exert on transforming deoxyribonucleic acid (DNA) of *Bacillus subtilis* a predominantly mutagenic effect at high (≥ 1 M) and a predominantly inactivating effect at low ($<10^{-1}$ M) HA concentrations. The inactivating effect is indirectly caused by some product of the reaction between HA and O_2 which apparently produces nitroxyl (HNO), hydrogen peroxide (H_2O_2), and several intermediate radicals. The formed peroxide is destroyed again by a further reaction with HA. Both the production of peroxide from HA and the inactivation of transforming DNA by HA are absent without oxygen and are inhibited by the addition of EDTA, pyrophosphate, KCN, $FeCl_3$, catalase, or peroxidase. The same agents also inhibit DNA inactivation by H_2O_2. The inactivating effect therefore seems to be caused by radicals formed during the production and destruction of peroxides by HA, and perhaps, in addition, by nitroxyl.

The mutagenic effect does not depend on oxygen and is not inhibited by pyrophosphate. It involves the direct reaction of HA groups with cytosine. These results explain earlier observations on the HA concentration dependence of inactivation and mutation and on the induction of chromosomal breaks.

Spontaneous or induced chemical changes of deoxyribonucleic acid (DNA) can be subdivided, with regard to their biological effects, into predominantly mutagenic and predominantly inactivating alterations. The former, which we shall briefly call "mutagenic alterations," involve changes of one (or few) nucleotide pairs without preventing DNA duplication. Depending on the DNA region, the particular growth conditions, and the screening methods such alterations can produce mutations, be lethal, or escape detection. Predominantly inactivating alterations of DNA, which we shall briefly call "inactivating alterations," usually are more drastic changes of one or more DNA bases, cross links, or breaks of DNA strands; they usually prevent the normal DNA duplication to advance beyond the altered site except when the alteration is repaired. Inactivating alterations can initiate chromosomal breaks and aberrations which either are lethal to the cell or occasionally survive, in which case they may be detected as mutation or have no apparent phenotypic effect.

For cells, it is usually difficult to distinguish inactivating changes of DNA from lethal changes of other cell components. This problem is eliminated by the use of transforming DNA for which inactivating alterations prevent transformation (for reasons that may differ for different agents).

We have utilized the transforming system to analyze both the mutagenic and the inactivating effects of hydroxylamine (HA),[1] *N*-methylhydroxylamine (*N*-methyl-HA), and *O*-methylhydroxylamine (*O*-methyl-HA). Hydroxylamines, containing a free -NOH group, exhibit at high concentrations (1 M) a mutagenic effect (for phages: Freese *et al.*, 1961a,b; Schuster and Vielmetter, 1961; Champe and Benzer, 1962; for transforming DNA: Freese and Strack, 1962; Strack *et al.*, 1964) and at low concentrations ($\leq 10^{-2}$ M) an inactivating effect (Freese *et al.*, 1961a; Freese and Freese, 1964). The mutagenic effect has been explained by the direct action of HA on cytosine (Freese *et al.*, 1961a,b; Schuster, 1961; Brown and Schell, 1961, 1965; Verwoerd *et al.*, 1961; Brown and Phillips, 1965). In contrast, we shall show that the inactivating effect is indirectly caused by a reaction product of HA groups with oxygen.

Materials and Methods

Chemicals. $NH_2 \cdot HCl$, platinum black, and ascorbic acid were obtained from Fluka Chemical Co., Buchs, Switzerland; *N*-methyl-HA·HCl from Aldrich Chemical Co., Milwaukee, Wis. (for purity see Freese and Freese, 1964); nitrogen, low in oxygen content, from Matheson Co., East Rutherford, N. J.; H_2O_2 (30% solution) from Merck; Tris from Sigma Chemical Co.; all other chemicals from Fisher Scientific Co., Silver Spring, Md. $FeCl_3$ was kept at 0.1 M in 0.01 N HCl and then diluted into the reaction mixture just before the experiment started. DNA of prototroph *Bacillus subtilis* 60009 was prepared as described earlier (Freese and Freese, 1963).

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[1] Abbreviations used in this paper: HA, hydroxylamine.

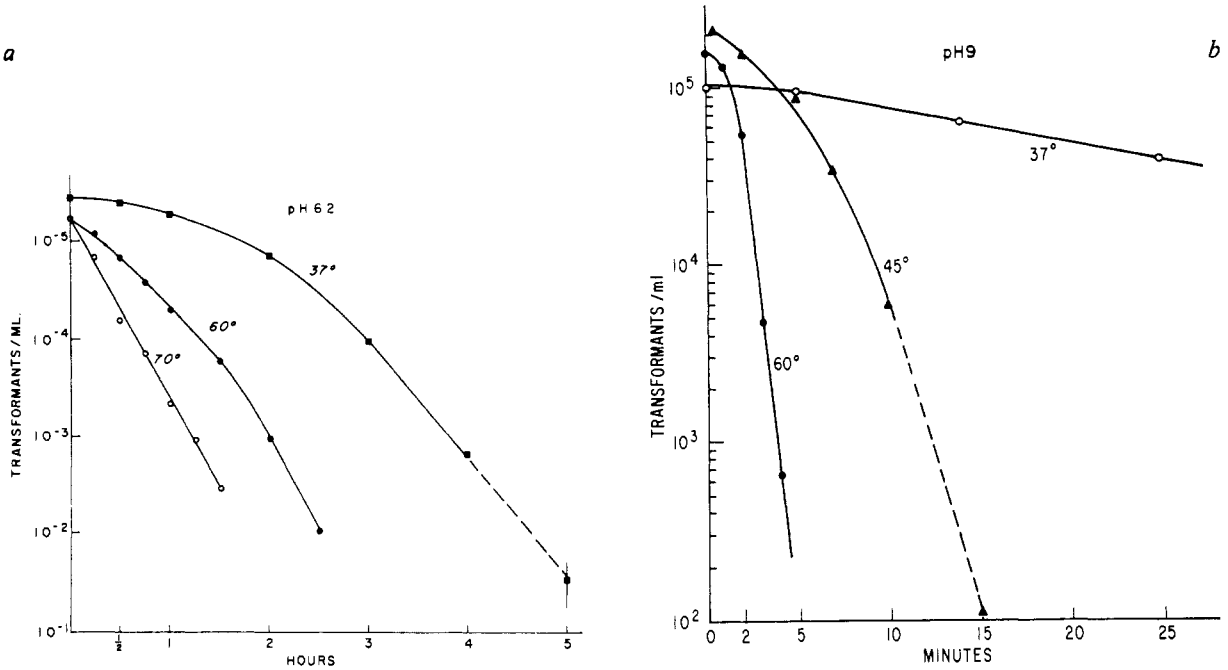

FIGURE 1: Inactivation of the tryptophan marker of *B. subtilis* transforming DNA by 10^{-2} M HA. (a) In 0.1 M sodium phosphate at pH 6.2; (b) in 0.05 M sodium carbonate at pH 9.0.

Treatment of Transforming DNA. B. subtilis DNA (≈1 mg/ml) in 2 M NaCl was usually diluted 10-fold in 2 M NaCl and then again 10-fold in the reaction mixture. For the experiments using different DNA concentrations the sodium concentration in the reaction mixture was adjusted by NaCl to remain always the same as in the standard procedure. Buffers and pH values of the reaction mixtures are stated for each experiment. pH was always adjusted by NaOH. Except when specially stated, 0.05 ml of DNA was added to 0.45 ml of the freshly prepared ice-cold reaction mixture in a 13 × 100 mm tube, a control sample was taken, the tube immediately was placed in the water bath of the employed temperature, and then stoppered. At different times, 50-μl aliquots were removed into 2.5 ml of an ice-cold stopping mixture. The DNA was 10-fold further diluted in the transformation experiment.

Stopping mixture consisted of 0.05 M Tris, pH 7.5, + 1 M NaCl + 10% acetone. For experiments using H_2O_2 5 μg/ml of catalase was added.

The transformation procedure employed as recipient the tryptophan⁻ strain *B. subtilis* 60 087. Transformation to tryptophan independence and production of fluorescent colonies were assayed as described previously (Freese and Strack, 1962).

The *oxygen tension* was measured by an oxygen electrode using a mixing chamber and a potentiometer set up as described by Kielley (1963). The change in voltage after HA addition was followed by a 10-mv recorder.

The *hydrogen peroxide* determination was performed similar to the method described by Egerton *et al.* (1954). Titanium sulfate (300 mg) was dissolved in 80 ml of water plus 20 ml of concentrated H_2SO_4 in the presence of nitrogen. The insoluble residue was filtered off by a glass fritte. This solution was directly used for the assay, without further reduction. The H_2O_2 solution (3 ml) was rapidly mixed with 1 ml of the titanium sulfate reagent and the OD_{410} was determined within 30 min. The extinction increased linearly with the H_2O_2 concentration up to about 1.5×10^{-3} M H_2O_2 ($OD_{410} \approx 0.8$). No color formation was observed with freshly prepared HA (pH 6–9) at any concentration, or with sodium hyponitrite (*trans* form), nitrite, nitrate, or with NO gas. No interference of the color reaction with H_2O_2 was observed with HA (10^{-2} M), EDTA (10^{-2} M), sodium pyrophosphate (5×10^{-2} M), KCN (10^{-2} M), or $FeCl_3$ (2×10^{-3} M, which added its own color to that of the titanium–H_2O_2 complex). The color developed immediately and was stable for at least 1 hr.

After prolonged reaction of HA at pH ≥9, a compound was produced which interfered with the titanium sulfate color assay since the yellow color was formed only initially and vanished after about 1 min at room temperature. This effect was probably due to nitrous acid, which is produced during the oxidation of HA and which does react with H_2O_2 at low pH. This reaction could be observed even after a yellow complex between titanium sulfate and H_2O_2 had been formed, because the color rapidly vanished when a small amount of sodium nitrite was added. In order to enable the determination of H_2O_2 even under these conditions, urea (0.4 M) was added to the titanium sulfate reagent (urea reacts immediately with nitrous acid). This addition did not affect the normal color development but prevented the fading of color observed before. In order to obtain rapid complete acidification, 1 ml of the test

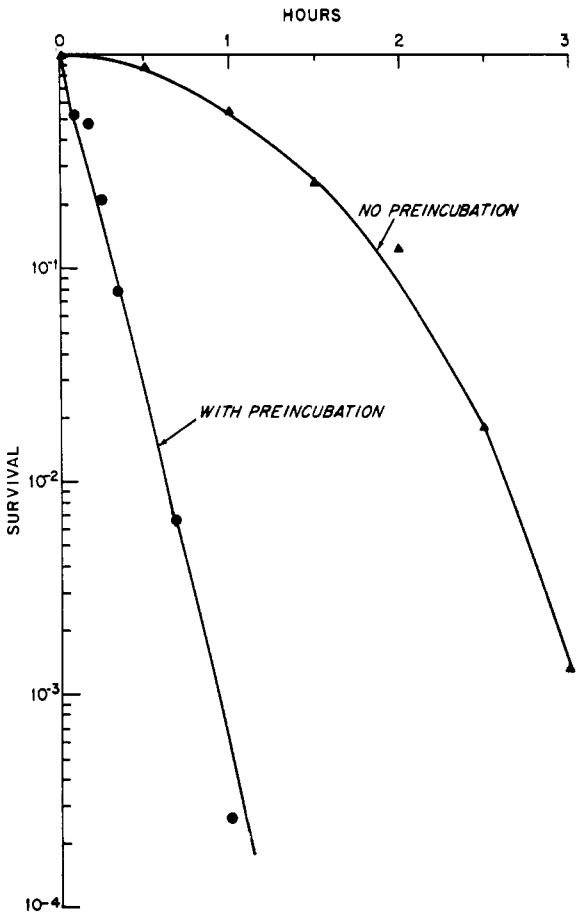

FIGURE 2: Effect of preincubation on the inactivation of transforming DNA by 10^{-2} M HA in 0.05 M sodium phosphate at pH 7.5. No preincubation: DNA added to freshly prepared HA solution and inactivation followed at 45°. With preincubation: HA solution preincubated for 2 hr at 45°, then 0.1 ml of DNA was added to 0.9 ml of solution and inactivation followed at 45°.

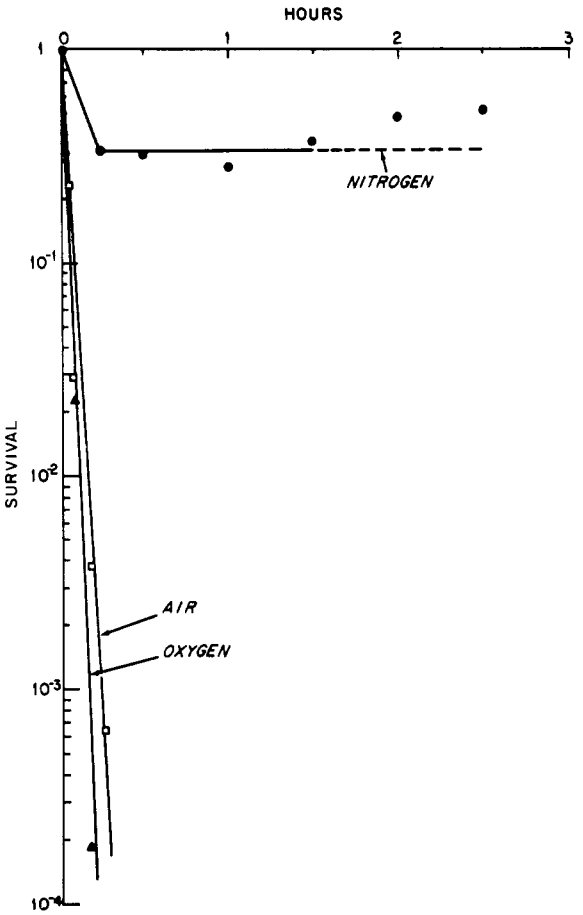

FIGURE 3: Effect of oxygen deficiency on the inactivation of transforming DNA by 10^{-2} M HA in 0.05 M sodium phosphate, pH 7.5, at 45°. The ice-cold HA mixture without DNA was gassed for 30 min. To 0.9 ml, 0.1 ml of gassed DNA was added and the inactivation followed.

solution was rapidly mixed (on Vortex) with 1 ml of the titanium sulfate–urea reagent. The OD_{410} was read within 30 min.

HA determination by quinolinol employed essentially the method of Frear and Burrell (1955) except that trichloroacetic acid and $MnCl_2$ were left out. HA determination by nitrite employed a combination of the methods of Csaky (1948) and Spencer *et al.* (1957). *Nitrite* was determined by the method of Spencer *et al.* (1957).

Results

Reactions of Transforming DNA with Hydroxylamines

Time Course of Inactivation. Prototroph DNA of *B. subtilis* was treated by HA in different buffer solutions at different temperatures and aliquots were diluted 50 times into an ice-cold "stopping mixture" at different times. A constant amount of each aliquot was then used for transformation to tryptophan independence. Inactivation refers here to the decrease in the frequency of $tryp^+$ transformants. At 75° and pH 6.2, the log of survival (log B/B_0) decreased linearly with time for all HA concentrations (Freese and Freese, 1964) (B = titer of transformants after treatment of DNA for time t, and B_0 = titer at time 0). At lower temperatures, however (and HA concentrations $<10^{-1}$ M), the inactivation rate $k = -(d/dt) \ln B$ (t in hours) increased initially ("acceleration period") and became constant only after some time of treatment. This can be seen in Figure 1 for 10^{-2} M HA. The inactivation rates increased with both pH (at least up to pH 10) and temperature.

On superficial inspection, the curves in Figure 1 might suggest a multi-hit phenomenon of inactivation. But the increasing straightness of the curves with increasing temperature would then imply that the number of hits (*i.e.*, chemical reactions) needed to inactivate would decrease with increasing temperature until, at 75°, one hit would suffice. We want to consider therefore two other explanations of the curves in Figure 1.

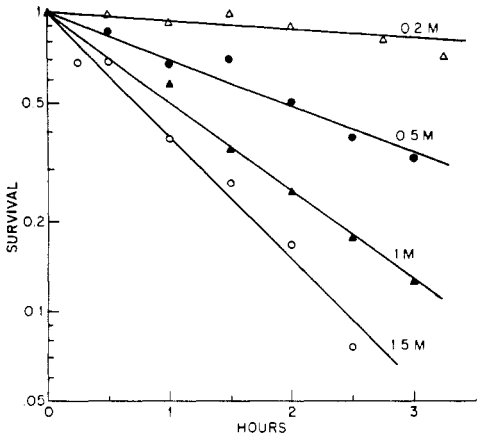

FIGURE 4: Inactivation of transforming DNA by different concentrations of HA in nitrogen; 0.05 M sodium phosphate, pH 6.2, at 75°. Nitrogen was bubbled through the mixture for 30 min at 75° before DNA was added. The remaining operation was performed as described in Figure 3 but no gas was ever blown into the reaction mixture. Tubes were stoppered and nitrogen was circulated only just before and after samples were taken.

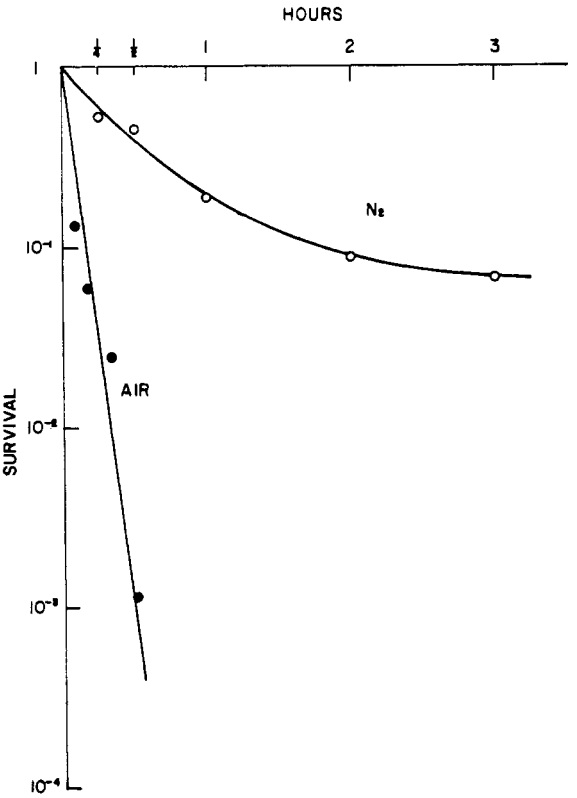

FIGURE 5: Effect of oxygen deficiency on the inactivation of transforming DNA by 10^{-2} M *N*-methyl-HA in 0.05 M sodium phosphate, pH 6.2. Experimental conditions as in Figure 3, but 75°.

HA might attach to DNA without inactivating it such that the attached molecules would inactivate DNA only subsequently. The acceleration period would then represent the time during which an increasing number of DNA sites would be covered by HA until all reactive sites were saturated.

Alternatively, not HA itself but some other reagent made from HA might cause the inactivation. Its amount would increase during the acceleration period until eventually the reagent would attain a stationary state concentration determined by the competition between its production and destruction.

Effect of Preincubation of the HA Solution. In order to decide between these alternatives we changed the reaction conditions slightly. In the foregoing experiments we had added DNA to the freshly prepared HA mixture and immediately incubated it in the reaction bath. Now we preincubated the HA mixture (10^{-2} M, pH 6.2) at 45° for 2 hr before adding the DNA in order to measure its inactivation at 45°. Figure 2 shows that in the preincubated HA mixture a linear inactivation curve was obtained immediately. A gradual increase of the inactivation at 45° was also observed when a 10^{-2} M HA solution of pH 9 (0.1 M sodium borate) was preincubated at 70°. Preincubation for 0, 20, and 60 min gave initial inactivation rates of 3, 18, and 39, respectively. The inactivation of DNA must therefore be caused by some reagent R produced in the dilute HA solution.

The Oxygen Effect of Inactivation by HA. How is the active reagent (R) produced from HA? R could not be an ionic species of HA since that would be in equilibrium with HA practically immediately. Nor could any particular buffer be necessary for the formation of R since inactivation curves of the type shown in Figure 1 were observed for carbonate, phosphate, and Tris buffer. Hence only two chemically reasonable possibilities remained: (1) R was produced by a reaction of HA with itself, or (2) R was produced by a reaction of HA with oxygen.

A. To determine the relative effect of air, nitrogen, and oxygen, the gases were bubbled for 30 min through the ice-cold reaction mixture containing 10^{-2} M HA (0.05 M sodium phosphate, pH 7.5) but no DNA. All tubes, pipets, and the DNA were gassed before use. DNA (0.1 ml) was then added to 0.9 ml of reaction mixture and the tube was transferred to a 45° bath. Aliquots (50-μl) were taken into 2.5 ml of ice-cold stopping mix. As Figure 3 shows, the transforming activity rapidly decreased in air or oxygen (the gas pretreatment eliminated the inactivation lag). In the presence of nitrogen, however, the transforming activity remained essentially constant. The small initial decrease probably resulted from a remainder of oxygen or an oxidation product of HA. The later increase of transformants can be attributed to evaporation. Both complications have been avoided in subsequent experiments (see Figure 4). The results in Figure 3 show that the reagent R is produced mainly by a reaction of HA with oxygen.

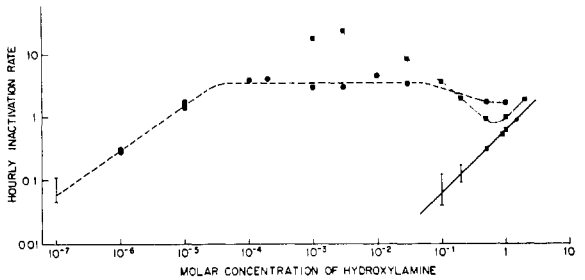

FIGURE 6: HA concentration dependence of the inactivation rates of transforming DNA in air and nitrogen at 75°. The pH was adjusted to 6.2 by NaOH. In air: --------- = 0.02 M sodium phosphate + HA·HCl; ····· = same plus NaCl to give a total of 3 M Na^+. In nitrogen: ——— = 0.05 M sodium phosphate + HA·HCl. Conditions in N_2 as in Figure 4.

B. THE OXYGEN EFFECT FOR *N*-METHYL-HA. The inactivating effect has also been observed for HA derivatives such as *N*-methyl-HA (Freese and Freese, 1964). Figure 5 shows that this effect (at 10^{-2} M *N*-methyl-HA, pH 7.5, and 75°) decreased greatly when air was replaced by nitrogen. Since the inactivation curve eventually leveled off, the initial decline was probably caused by some residual oxygen or an oxidation product of *N*-methyl-HA in the reaction mixture.

The HA Concentration Dependence of Inactivation in Nitrogen. The oxygen effect has so far been determined at 10^{-2} M HA. We now wanted to discover whether oxygen was also required for DNA inactivation at high HA concentrations. Since we had earlier reported the rates of DNA inactivation at different HA concentrations for conditions of air being present (Freese and Freese, 1964), we repeated these measurements in the presence of nitrogen but used otherwise the same conditions (pH 6.2, 75°). The reaction mixture was prepared by diluting a 1 M HA solution (pH 6.2), which contains practically no R, into 0.05 M sodium phosphate buffer at pH 6.2 that had been deaerated and saturated with nitrogen. This solution was further treated with nitrogen for 30 min at 75°, whereupon 0.1 ml of nitrogen-treated DNA was added to 0.9 ml of this solution and 50-μl samples were withdrawn at different times. The tubes were stoppered and deeply immersed in the bath. Before, during, and after withdrawing a sample nitrogen was blown into the tube. Figure 4 displays the inactivation curves whose slopes increased with the HA concentration. The inactivation rates, solid line in Figure 6, increased approximately linearly with the HA concentration in contrast to the rates observed in air (dashed and dotted lines) but similar to the mutation rates in air (Freese and Freese, 1964). In order to check that this oxygen-independent effect was actually mutagenic the production of fluorescent mutants was assayed for 1 M HA. Figure 7 shows a strong mutagenic effect whose rate was about 2 times higher than that observed in air.

DNA Dependence of Inactivation Rates. When at pH 6.2 the reaction temperature was sufficiently high (*e.g.*,

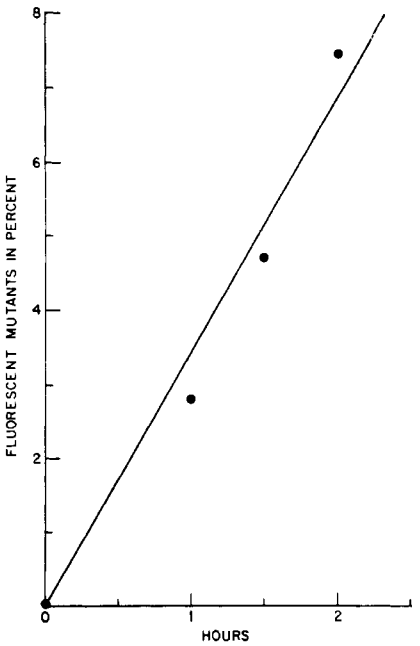

FIGURE 7: Induction of fluorescent mutations, linked to the tryptophan synthetase gene, by 1 M HA, in nitrogen; 0.05 M sodium phosphate, pH 6.2, at 75°. Conditions as described in Figure 4.

75°) or the preincubation time sufficiently long, the inactivation rates in air did not appreciably change with time even for low HA concentrations. This shows that the inactivating reagent R must be not only continuously produced (from HA and oxygen) but also continuously destroyed (by further reaction with HA or DNA). The stationary state concentration of R depends therefore on the concentration of HA, oxygen, and DNA (and any catalysts or other factors influencing the reaction rates). At low DNA concentrations, the concentrations of HA and O_2 determine the stationary state concentration of R, whereas at large DNA concentrations the reaction of R with DNA might dominate the rate at which R is destroyed and therefore the stationary state concentration of R.

In order to determine the effect of the DNA concentration on inactivation rates, we measured them at different concentrations of HA and DNA (at 75°, pH 6.2). The result in Figure 8 shows that at low DNA concentrations the inactivation rates were independent of the DNA and HA concentrations. However, when the DNA concentration exceeded a "critical value" the inactivation rate decreased. The critical DNA concentration itself increased with the HA concentration.

The Effect of Different Agents on DNA Inactivation by HA. All of the following experiments were done in 0.05 M sodium phosphate, pH 7.5, at 45°. All agents were adjusted to this pH. (a) Reducing agents such as ascorbic acid, mercaptoethanol, or sodium hydrosulfite retarded the inactivation when they were added to a 10^{-2} M HA solution (see Figure 9). (b) The addition of

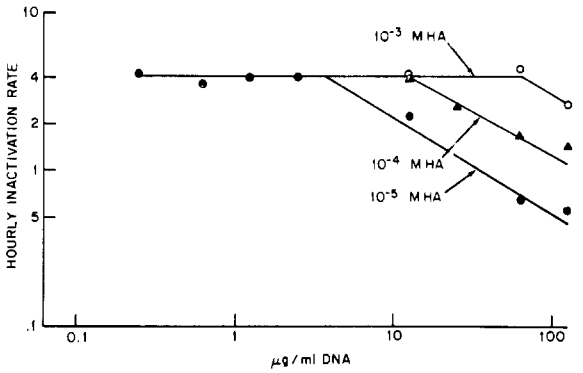

FIGURE 8: DNA concentration dependence of inactivation rates at different HA concentrations; 0.02 M sodium phosphate, pH 6.2, at 75° in air. The final concentration of DNA in the transformation tube was adjusted to 0.02 μg/ml or less.

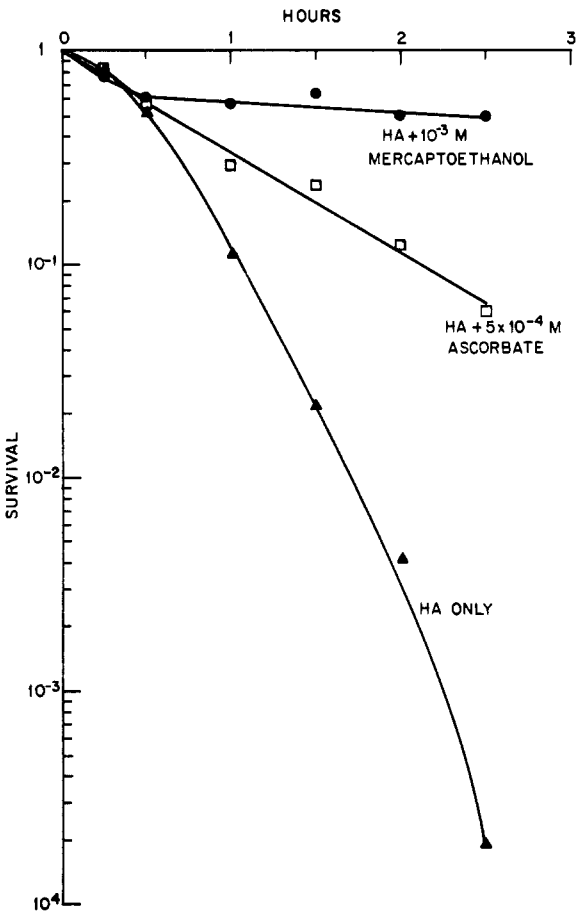

FIGURE 9: Effect of reducing agents on the inactivation of transforming DNA by 10^{-2} M HA in 0.05 M sodium phosphate, pH 7.5, at 45°.

2×10^{-4} M $FeCl_3$ (Figure 10b) or a small amount of platinum black (Figure 10a) to a 10^{-2} M HA solution gave rise to a linear inactivation curve. These agents obviously kept the concentration of reagent R at a stationary level. In 2×10^{-3} M $FeCl_3$ inactivation was nearly completely suppressed (Figure 10b). (Although $Fe(OH)_3$ precipitated, an inhibitory concentration of Fe^{3+} apparently remained in solution.) (c) When EDTA (10^{-2} or 10^{-3} M) was added to a 10^{-2} M HA solution, a linear inactivation curve was observed, indicating that EDTA kept the concentration of reagent R at a stationary level (Figure 10a). (d) Both sodium pyrophosphate (0.05 M) and KCN (10^{-2} M) prevented the inactivation of DNA by 10^{-2} M HA nearly completely (Figure 10a). (e) Catalase (100 μg/ml) and peroxidase (100 μg/ml) completely prevented the inactivation of DNA by 10^{-2} M HA (Figure 10b).

Combination of HA and H_2O_2. The combination of HA (10^{-2} M) and H_2O_2 (2×10^{-4} M) produced a much more rapid inactivation than either of the two agents separately or even the additive effect that would result if the two agents acted independently (see Figure 11a). In the presence of EDTA (10^{-2} M) the combination of HA and H_2O_2 produced nearly the same initial inactivation rate as without EDTA, whereas the separate effect of H_2O_2 was completely and that of HA was appreciably inhibited by EDTA (see Figure 11b). This indicates that the combined effect, in contrast to the separate effects, did not depend on the presence of trace metals. After some reaction time the inactivation rate of the combined effect decreased faster in the presence than in the absence of EDTA, as if with EDTA H_2O_2 was used up more rapidly. This agrees with the chemical findings (see the discussion on H_2O_2 destruction by HA below).

Pyrophosphate and KCN completely inhibited both the separate and the combined effects (Figure 11b), which suggests that they inhibit the combined effect by a different mechanism than EDTA.

Elimination of Nitrous Acid as the Active Reaction Product. One of the secondary reaction products of HA and oxygen is nitrous acid. In order to rule out that it is the inactivating agent R, we treated DNA (in 0.05 M sodium phosphate, pH 7.5, at 75°) in the presence and absence of nitrogen with 2×10^{-4} M sodium nitrite. Within 2 hr at 75° the survival was still 3.8×10^{-1}, in contrast to the effect of 10^{-4} M HA for which, under the same conditions but only at 45°, the survival was 2.3×10^{-4} in 2 hr.

Chemical Results

Oxygen Consumption by HA Solutions. The consumption of oxygen by HA solutions was measured in 0.05 M sodium phosphate (pH 7.5, 45°) by the decrease of oxygen tension recorded after addition of a concentrated HA solution (same buffer and pH). Figure 12 shows how the initial rates of oxygen decrease $[-(d[O_2]/dt)/[O_2](\text{initial})]$ increased with the HA concentration.

The oxygen tension exponentially approached a stationary state between consumption by HA and uptake from air. The near-equilibrium values, observed after 12 min, are also plotted against the HA concentration in Figure 12. At high HA concentrations the

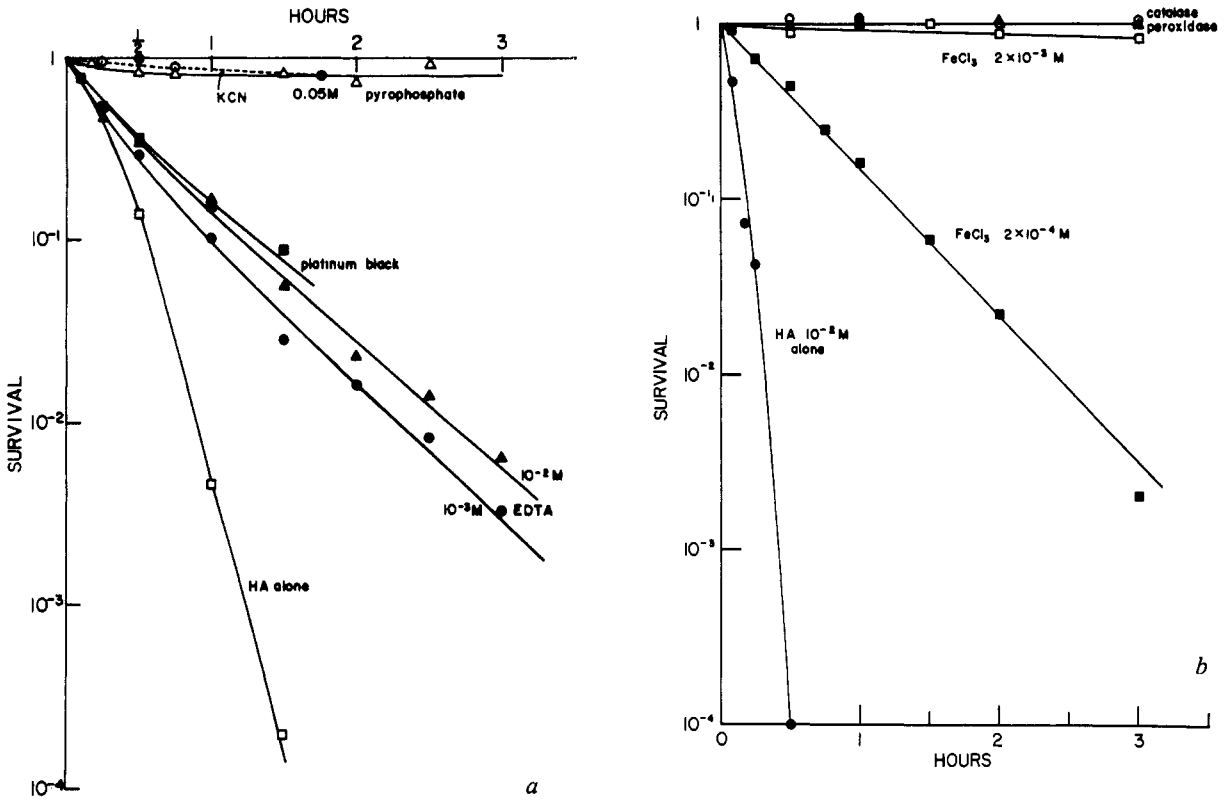

FIGURE 10: Effect of various agents on the inactivation of transforming DNA by 10^{-2} M HA in 0.05 M sodium phosphate, pH 7.5, at 45°. The same result was obtained irrespective of whether the agent was added to the dilute HA solution or mixed with a 1 M HA solution and then diluted. All reagents were adjusted to pH 7.5, except $FeCl_3$, which was kept as 0.1 M solution in 10^{-2} M HCl and diluted into the reaction mixture. Catalase and peroxidase were both 100 μg/ml.

stationary state concentration of O_2 was greatly reduced.

Peroxide Production by HA Solutions. A 10^{-2} M solution of HA at pH 8.5 or 9 (stirred in the presence of air) produced a compound which upon acidification in the titanium sulfate reagent gave rise to a yellow complex that is typical for hydrogen peroxide (Egerton *et al.*, 1954) (see Figure 13). This compound could be either H_2O_2 or some peroxide that liberates H_2O_2 upon acidification, or both. In the following we shall briefly name it "peroxide."

No peroxide production was observed at pH 7.5 or 6.2 for any HA concentration at 45 and 70° for at least 2 hr; the peroxide concentration must therefore have stayed below 10^{-4} M (the limit of detectability by the titanium sulfate method).

N-Methyl-HA also gave rise to peroxide, whereas *O*-methyl-HA did not (Figure 13).

When oxygen was removed by evacuation and nitrogen was bubbled through the HA solution, no peroxide was produced (see Figure 13).

The peroxide concentration, observed after some time of incubation, depended on the HA concentration as shown in Figure 14. For these experiments test tubes containing different concentrations of HA (pH 8.5) stood, or were aerated, at 70° and samples were withdrawn at different times for peroxide determination. Aeration greatly increased the concentration of peroxide produced. But the highest concentration of peroxide was found at an intermediate HA concentration, both with and without aeration, after 30, 60, and 90 min incubation.

In 1 N NaOH the oxidation of HA produces an unstable yellow compound with an absorption maximum of 302 mμ (Yagil and Anbar, 1964). This compound has been tentatively identified as peroxynitrous acid (ONOOH) which can also be produced as a transient material by the reaction of nitrous acid and H_2O_2 at acidic pH (Gleu and Hubold, 1935) and is stabilized by high pH (Halfpenny and Robinson, 1952).

We have verified that a 10^{-2} M HA solution in 0.2 N NaOH produced (at 45 or 70°, with intense aeration) a compound with an absorption maximum of 302 mμ. Its concentration increased (*e.g.*, at 45° for 60 min to $OD_{302} = 4$) until the HA concentration had decreased below 5% (as determined by the quinolinol method); then the OD_{302} decreased (to $OD_{302} = 1$ in 230 min). The concentration of peroxides, detectable by the titanium sulfate–urea method, increased and decreased

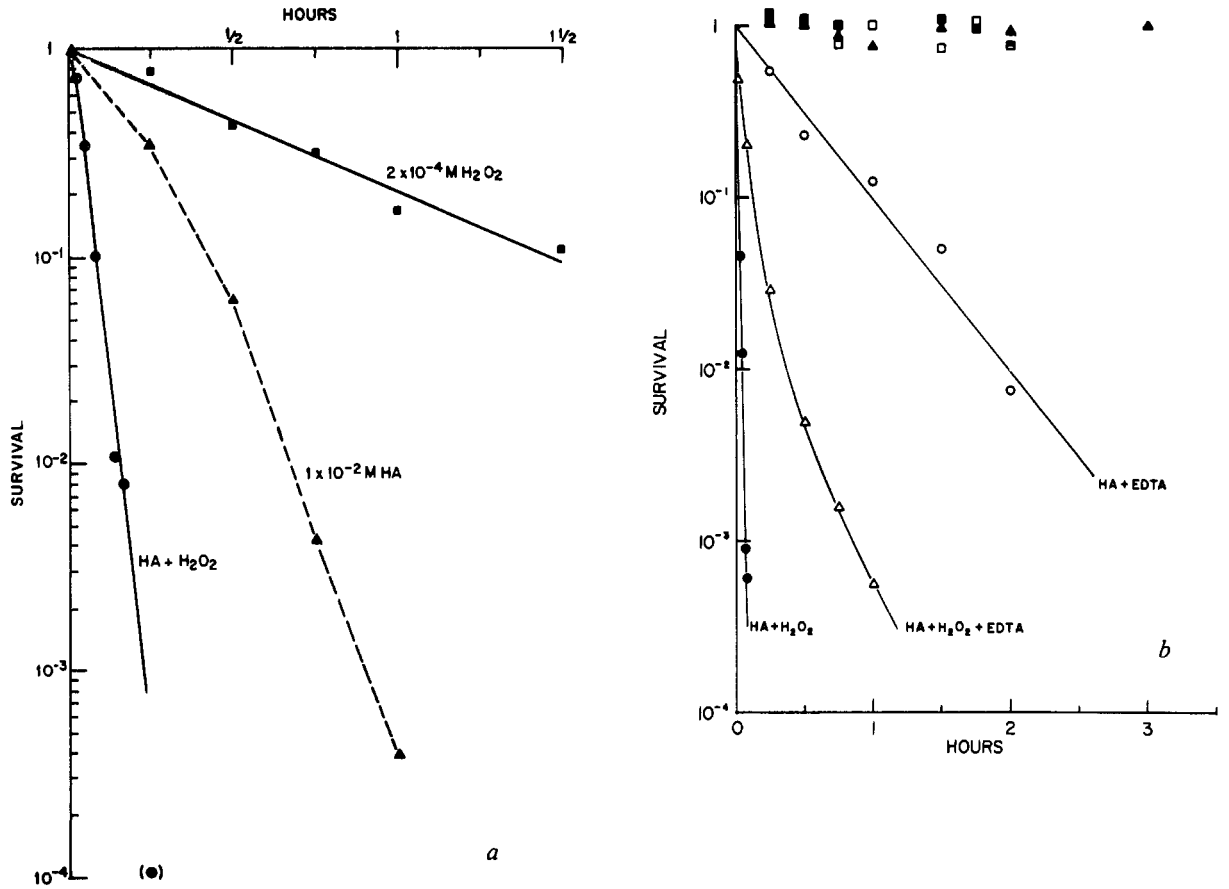

FIGURE 11: Inactivation of transforming DNA in 0.05 M sodium phosphate, pH 7.5, at 45°. (a) In 10^{-2} M HA and 2×10^{-4} M H_2O_2 separately and in combination; (b) in 10^{-2} M HA and 10^{-3} M H_2O_2 separately and in combination, together with different agents: ▲ = H_2O_2 + 10^{-2} M EDTA; ■ = HA + H_2O_2 + 10^{-2} M KCN; □ = HA + H_2O_2 + 5×10^{-2} M sodium pyrophosphate.

strictly parallel to the OD_{302} and was about equal to the concentration of peroxynitrite, if one assumes for the latter the extinction coefficient of 1300 (Yagil and Anbar, 1964).

When 10^{-2} M HA was aerated at pH 9 (in 0.1 M borate or carbonate), however, no peroxynitrite could be detected by its OD_{302} absorption, even after addition of NaOH to 0.2 N. At the same time the concentration of peroxide increased as shown in Figure 15. When an aliquot of this solution was rendered 0.2 N in NaOH at any time after peroxide had been formed, the extinction at 302 mμ increased for about 2 min and then slowly decreased again. The spectrum of the transient compound had an absorption maximum below 205 mμ and no absorption peak between 205 and 450 mμ. This compound was also produced when fresh HA was added to H_2O_2 in 0.2 N NaOH, as will be explained below. The peroxide, produced by the oxidation of HA at pH 9, was therefore probably mainly H_2O_2 and definitely not peroxynitrite. Since at the same time nitrite was produced (see below), it is possible that both compounds resulted from the hydrolysis of peroxynitrite as an unstable intermediate.

Effect of Different Agents on the Peroxide Production. In the presence of EDTA (10^{-3} M) or $FeCl_3$ (2×10^{-4} M) no peroxide development was observed by 10^{-2} M HA at pH 8.5 (see Figure 16). At 45°, *e.g.*, no peroxide was observed for 5 hr. These compounds did not affect the yellow complex formation by the titanium sulfate reagent with either an H_2O_2 solution (10^{-3} M) or with a preincubated HA solution.

Sodium pyrophosphate (0.05 M) or potassium cyanide (10^{-2} M) also partially inhibited the production of peroxides by 10^{-2} M HA (see Figure 16). But after long times of treatment the peroxide concentration even exceeded that found in an HA solution alone, probably because the destruction of peroxide by HA was even more inhibited than its production (see below). Neither compound interfered with the staining reaction between H_2O_2 and the titanium sulfate reagent.

6-Mercaptoethanol (2×10^{-3} M) prevented the development of titanium sulfate stainable material by 10^{-2} M HA. Mercaptoethanol also eliminated H_2O_2 itself from a solution.

Catalase (30–50 μg/ml) initially inhibited the production of peroxides by 10^{-2} M HA, pH 9, at 45° (see

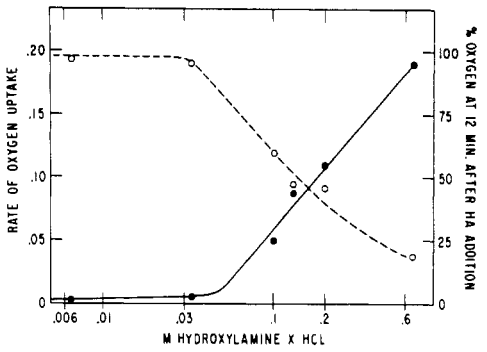

FIGURE 12: Oxygen consumption by hydroxylamine. The oxygen tension was measured by an oxygen electrode in 0.05 M sodium phosphate at pH 7.5. After a stationary state had been obtained, a concentrated HA solution (same buffer, pH 7.5) was added and both the initial rate of oxygen uptake $[-(d[O_2]/dt)/[O_2](initial)]$ = --------- and the oxygen concentration after 12 min (which was close to the new equilibrium) = ——— were determined.

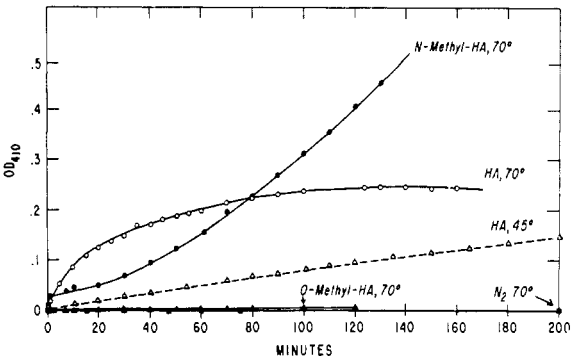

FIGURE 13: Peroxide production by hydroxylamines; 300 ml of 0.1 M sodium borate, pH 8.5 (or 9 when stated), were stirred in a 1-l. beaker (or in the case of nitrogen deaerated and N_2 treated in an erlenmeyer) at 70°; 3 ml of a 1 M buffered solution of the hydroxylamines was added and aliquots were removed at different times for the determination of H_2O_2 by the titanium sulfate reagents.

Figure 15). Very soon, however, peroxide appeared in the medium, apparently because catalase could not remove it fast enough. HA actually inhibits catalase (Nicholls, 1964), as was immediately apparent when catalase was added to a 5% H_2O_2 solution, buffered at pH 7.5 or 8.5. In the absence of HA the solution extensively liberated oxygen and showed no reaction with the titanium sulfate reagent after 10 min at room temperature. In contrast, when the H_2O_2 solution contained HA (0.2 M), only a few gas bubbles appeared slowly and a strong yellow color was produced with titanium sulfate. The results are quantitatively expressed in Table I. It is thus clear that, in the presence of HA, catalase can slowly eliminate H_2O_2 only when the concentration of HA is low and H_2O_2 is produced at a low rate. The concentration of peroxide eventually reached the same level as in HA alone, apparently because catalase activity was destroyed (the same occurred much more rapidly at 70°).

TABLE I: Effect of Catalase and Peroxidase on the Concentration of H_2O_2.[a]

Agents Added to 10^{-3} M H_2O_2	Extinction at 410 mμ: Time after Addn of Last Compd: Immediately	10 min	20 min
0.02 M HA	0.52	0.51	
Catalase, 3.0 μg/ml	0.01	0.01	
First HA, then catalase	0.51	0.47	
First 10 min catalase, then HA	0.005	0.005	
Peroxidase, 30 μg/ml	0.53	0.52	0.52
First HA, then peroxidase	0.44	0.29	0.01
First 10 min peroxidase, then HA	0.51	0.30	0.01

[a] Each reaction mixture contained 10^{-3} M H_2O_2 and 0.1 M sodium borate, pH 9, and was incubated at room temperature. H_2O_2 was assayed by the titanium sulfate reagent, using the extinction at 410 mμ.

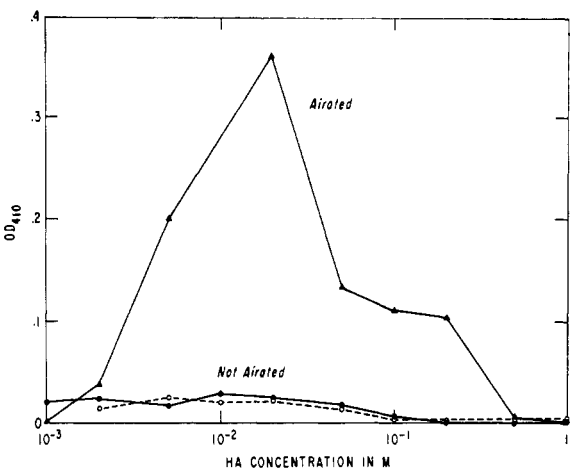

FIGURE 14: Dependence of peroxide production on the HA concentration, after 30-min incubation of the 10-ml mixture in wide test tubes at 70°; HA·HCl in 0.1 M sodium borate, adjusted to pH 8.5 by NaOH; ▲ = with aeration; ● = without aeration; ○ = without aeration, solution adjusted to a total concentration of 1 M NaCl.

Peroxidase (30 μg/ml) rapidly eliminated H_2O_2 in the

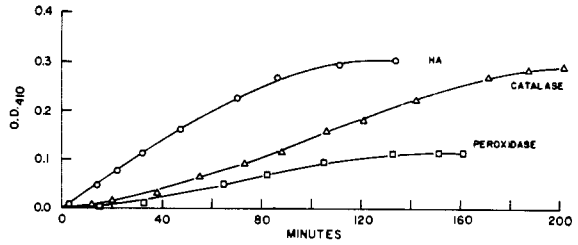

FIGURE 15: Peroxide production by 10^{-2} M HA in 0.1 M sodium carbonate, pH 9, at 45°, in the presence of catalase or peroxidase (each 50 μg/ml); 300 ml of the solution were efficiently aerated and 1-ml aliquots were removed for the titanium sulfate–urea assay.

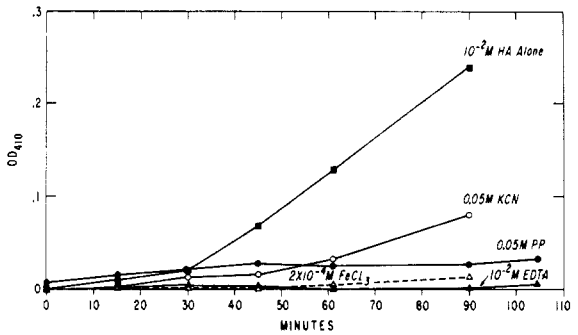

FIGURE 16: Peroxide production by 10^{-2} M HA and various additions; 50 ml of 0.1 M sodium borate, pH 8.5, plus various additions were incubated (without stirring) in a 250-ml beaker, at 70°; 0.5 ml of 1 M HA were mixed in and samples were withdrawn at different times for H_2O_2 determination.

presence of HA. Since peroxidase did not react with H_2O_2 alone, HA obviously served as an electron donor. This is quantitatively expressed in Table I.

In the presence of peroxidase (30–50 μg/ml) a 10^{-2} M HA solution (at pH 9) initially exhibited no peroxide (see Figure 15). Eventually, some peroxide was found but its concentration stayed below that observed for HA alone.

Other Reactions Followed in HA Solutions. The concentration of HA decreased in most of the foregoing biological and chemical experiments slowly enough that it could be assumed to be nearly constant. The rate of HA oxidation increased with both pH and temperature. In a typical solution of 10^{-2} M HA, pH 8.5, at 70°, the HA concentration decreased at a rate of 14%/hr when measured by the quinolinol method, and 9%/hr when measured by the nitrite method, which includes oxidated compounds such as HNO and HNO_2 (conditions as in Figure 13).

The oxidation of HA also produced nitrous acid whose concentration increased (in 10^{-2} M HA, pH 8.5, at 70°) at an initial rate of 6×10^{-5} M nitrous acid produced/hr (conditions as in Figure 13). The rate of HNO_2 production slowly increased.

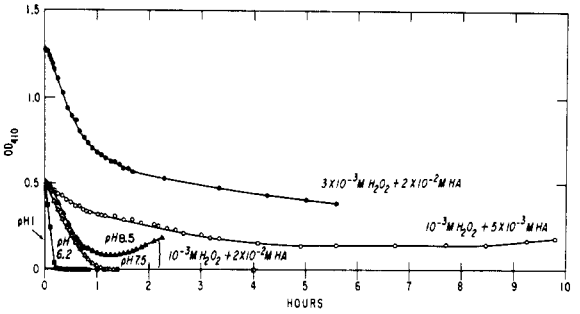

FIGURE 17: Destruction of H_2O_2 by incubation in the presence of HA at 70°; ●, ○, and ▲ = 0.1 M sodium borate, pH 8.5; △ = 10^{-3} M H_2O_2 + 2×10^{-2} M HA in 0.05 M sodium phosphate, pH 7.5; ■ = same in 0.05 M sodium phosphate, pH 6.2.

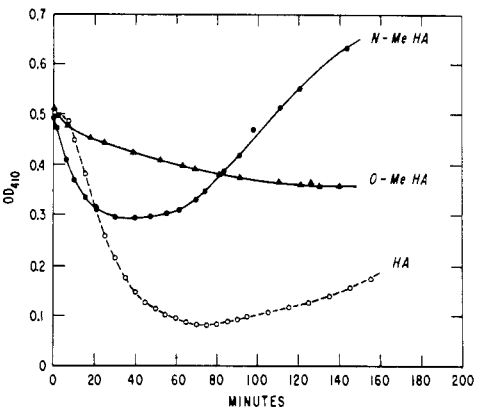

FIGURE 18: Destruction of H_2O_2 and production of peroxide by different hydroxylamines (2×10^{-2} M) in 0.1 M sodium borate, pH 8.5, at 70°. Initial concentration of H_2O_2 = 10^{-3} M. Experimental conditions as in Figure 17.

Hydrogen Peroxide Destruction by HA. HA reacted with H_2O_2 at basic, neutral, and slightly acidic pH values. At highly acidic pH the reaction rate (with 10^{-2} M HA) was slow enough that no significant change of the titanium sulfate–H_2O_2 color was observed within 1 hr at room temperature.

The decrease of H_2O_2 in the presence of HA is shown for the pH range 6.2–8.5 in Figure 17. At pH 7.5 or below the concentration of H_2O_2 decreased below a measurable value (<10^{-4} M). At pH 8.5, however, the titanium sulfate stainable material eventually increased again. This could either mean that some peroxide different from H_2O_2 increased in amount, or more likely that an equilibrium became established between the reaction product of HA·H_2O_2 and these compounds, such that the further production of H_2O_2 by oxidation of HA increased the concentration of H_2O_2. The establishment of such an equilibrium is also shown in the curve displaying the production of peroxide (H_2O_2) from HA (Figure 13).

TABLE II: Effect of Different Agents on the Destruction of 10^{-3} M H_2O_2 by 2×10^{-2} M HA in 0.05 M Sodium Phosphate, pH 7.5, at 70°.

Agent	Concn (M)	2×10^{-2} M HA Present	OD_{410} Initial	30 min	60 min
None	...	+	0.48	0.14	0.01
EDTA	10^{-2}	−	0.50	0.46	0.41
EDTA	10^{-2}	+	0.46	0.006	0.005
EDTA	5×10^{-2}	−	0.48	0.32	0.21
EDTA	5×10^{-2}	+	0.45	0.007	0.005
Sodium pyrophosphate	10^{-2}	+	0.67	0.64	...
Sodium pyrophosphate	5×10^{-2}	−	0.48	0.47	0.48
Sodium pyrophosphate	5×10^{-2}	+	0.47	0.47	0.49
KCN	10^{-3}	+	0.49	0.39	0.38
KCN	10^{-2}	−	0.51	0.47	0.46
KCN	10^{-2}	+	0.49	0.47	0.49

N-Methyl-HA also reacted with H_2O_2 and later showed the increased concentration of peroxide (see Figure 18). *O*-Methyl-HA, however, reacted with H_2O_2 only slowly and did not produce any additional peroxide.

H_2O_2 reacted with HA the faster the higher the HA concentration (see Figure 17). At 1 M HA and pH 6.2 or lower, the reaction was practically instantaneous.

Sodium pyrophosphate and potassium cyanide prevented the reaction of H_2O_2 with HA at pH 7.5 (Table II). $FeCl_3$ sometimes did and sometimes did not inhibit the reaction. This probably depended on the stage of $Fe(OH)_3$ precipitation which occurred very rapidly. EDTA, in contrast, even accelerated the reaction at pH 7.5 (see Table II).

The reducing agents Fe^{2+}, mercaptoethanol, ascorbate, naphthoquinone, and hydroquinone rapidly reacted with H_2O_2 whether HA was present or not.

In 0.2 N NaOH the reaction of HA with H_2O_2 could be followed spectrophotometrically. An ultraviolet-absorbing material appeared which had a broad absorption peak with a maximum below 205 mμ. In 5×10^{-4} M H_2O_2 + 10^{-2} M HA at room temperature, the extinction first increased for about 3 min and then decreased again, somewhat more slowly. The material obviously was unstable.

Discussion

Our results have shown that the inactivating effect of HA is oxygen dependent, whereas the mutagenic effect is not. In order to analyze the inactivating effect further we shall now analyze (1) which agents can be made during the oxidation of HA and how their production can be inhibited, and (2) which of these agents inactivate DNA and how the inactivation can be inhibited. This analysis enables us to explain the abnormal HA concentration dependence of DNA inactivation. We shall consider the relationship of these findings to the induction of chromosomal breaks, and finally we mention similar effects of other agents.

Chemistry of HA Oxidation. The oxidation of HA apparently proceeds by the over-all reaction

$$NH_2OH + O_2 \rightarrow HNO + H_2O_2 \qquad (1)$$

Nitroxyl has been isolated in the presence of liquid nitrogen (see Mellor, 1947; Gmelin, 1936) and as the sodium salt (Zintl and Harder, 1933). At room temperature the nonionized nitroxyl rapidly polymerizes to hyponitrous acid (HONNOH) (Mellor, 1947; Gmelin, 1936; Remy, 1956; Holleman and Wiberg, 1964), which disproportionates into nitrous oxide (N_2O) and water (Hughes and Stedman, 1963). HNO also reacts with HA to form nitrogen (Remy, 1956; Holleman and Wiberg, 1964). At high pH NO^- can apparently be oxidized to peroxynitrite ($ONOO^-$) (Yagil and Anbar, 1964), which at neutral or acidic pH may either hydrolyze to nitrite and H_2O_2 or rearrange (*via* radical decay into $\cdot NO_2$ + $\cdot OH$) into nitrate (Halfpenny and Robinson, 1952).

The formation of H_2O_2 is shown by the following results. (1) Oxidation of HA (at pH 9) produces a peroxide which upon acidification gives the color reaction typical for H_2O_2. (2) This peroxide is not peroxynitrite since it does not have an absorption peak at 302 mμ. (3) The material is eliminated, in the presence of HA, by reaction with peroxidase. (4) In 0.2 N NaOH the peroxide forms with HA a transient ultraviolet-absorbing compound which is also produced by a mixture of HA and H_2O_2. Most of the peroxide formed at pH 9 and below seems to be therefore H_2O_2.

Both the production of peroxide by HA and the inactivating effect of HA are inhibited by EDTA, Fe^{3+}, pyro-

phosphate, or CN^-. Since EDTA, pyrophosphate, and CN^- are known to complex metal ions, the peroxide formation probably is catalyzed by trace amounts of reduced transition metals. A catalytic effect of Cu^+, Fe^{2+}, etc., on the oxygen uptake by HA solutions at pH 9 has actually been measured by Moews and Audrieth (1959). The peroxide formation furthermore probably involves radicals which catalyze chain reactions similar to the autoxidation of aldehydes (Walling, 1957). Possible radical intermediates would be $\cdot OH$, $NH_2O\cdot$, $OHNHOO\cdot$, or, since the reaction is base catalyzed, some negatively charged radicals such as $\cdot NHO^-$ and $O\leftarrow\dot{N}HOO^-$. The potential scavenger action on radicals may therefore also explain the inhibitory effect of some of the above compounds, *i.e.*, Fe^{3+} (Walling, 1957), pyrophosphate (shown for polyphosphate; Butler and Conway, 1953), and CN^-.

The produced peroxide (H_2O_2) reacts further with HA at alkaline, neutral, and acidic pH values, probably giving rise to different final products (N_2O or N_2) depending on the pH. The reaction at pH 7.5 is inhibited by pyrophosphate and CN^-, but is even enhanced by EDTA. These findings can be explained in two ways. Either the reaction may require a trace metal catalyst which retains its catalytic activity when complexed with EDTA, but not when complexed with CN^- or pyrophosphate, or the radicals, needed for the reaction, may be trapped by pyrophosphate or CN^- but not by EDTA. In the latter case radicals would have to be formed without trace metals, as appears possible by the decomposition of H_2O_2 using the reducing power of HA (eq 2).

$$H_2O_2 + NH_3OH^+ \longleftrightarrow \cdot NH_2OH^+ + \cdot OH + H_2O \quad (2)$$

These two radicals, or at least the OH radical formed by the metal-catalyzed process, could accomplish the reaction between H_2O_2 and HA by the chain reactions shown in eq 3 and 4.

$$NH_3OH^+ + \cdot OH \longleftrightarrow \cdot NH_2OH^+ + H_2O \quad (3)$$

$$NH_2OH^+ + H_2O_2 \longleftrightarrow HNO + H_3O^+ + \cdot OH \quad (4)$$

The breakdown of H_2O_2 would be stopped by very low pH or by the accumulation of HNO, in agreement with our observations.

The Inactivating Reagent. Having enumerated the agents that can be produced by the oxidation of HA, we shall now determine which of them cannot and which can produce the inactivating effect.

The following oxidation products can be disregarded because they are known to leave DNA unaffected under the experimental conditions used here ($6.2 \leq pH \leq 9$): nitrous acid, nitrogen, and nitrous oxide.

Hyponitrous acid (HONNOH) occurs in two tautomeric forms (*cis* and *trans*). The *trans* form, which can be readily isolated, has been found by us to be inactive with respect to both DNA inactivation and mutation, at concentrations between 10^{-4} and 10^{-1} M (Freese and Freese, 1964). This renders also the *cis* form unlikely as the inactivating reagent because the tautomeric change from the *trans* to the *cis* form might be expected to occur frequently enough (at our reaction temperatures of up to 75°) to produce a concentration of 10^{-6} M *cis* in a 10^{-1} M *trans* solution; 10^{-6} M HA still exerts a significant inactivating effect on DNA (Freese and Freese, 1964).

One can eliminate hyponitrous acid as the inactivating agent also by a kinetic argument. Since at pH 6.2 and 75° the inactivation rate of DNA remained constant, within the concentration range of 10^{-4} to 2×10^{-2} M HA (Figure 6), the concentration of the inactivating reagent R must also have remained constant. In this range of zero-order concentration dependence the rates of production and destruction of R must therefore be proportional to the same power of the HA concentration. Both reaction products HNO and H_2O_2 satisfy this condition, because they both can be destroyed by treating with HA. The concentration of hyponitrous acid would then also show a zero-order concentration dependence on HA, because it is formed by dimerization of HNO and it is destroyed at a constant rate by disproportionation. Our inactivation rates in Figure 8 show that at sufficiently high concentrations of DNA the destruction rate of R decreases, obviously because this rate is then dominated by the reaction with DNA. If now R were hyponitrite, the decrease in the concentration should have no effect on the concentration of HNO itself since that is kept at equilibrium mainly by the HA-dependent production and destruction mentioned above. An increase of the HA concentration, within the zero-order concentration range, should therefore not alter the concentration of nitroxyl and thus the production rate of HONNOH, irrespective of the DNA concentration. Consequently, the inactivation rate should then not change with the HA concentration, even for high DNA concentrations. We have observed the opposite (see Figure 8): the decrease of inactivation rates observed for high DNA concentrations was clearly counteracted by increasing HA concentrations. Hyponitrous acid is therefore excluded as the inactivating agent.

The inactivating reagent still could be nitroxyl, a peroxide, or one or more of the radicals produced during the oxidation of HA. Radicals generally are very reactive and probably most radicals, formed during the oxidation of HA, can react with several components of DNA. In particular, OH radicals have been shown to inactivate DNA (Butler and Smith, 1950). Whereas the inactivating effect of radicals is without question, it is still open whether any of the nonradical compounds, whose concentration would be much higher than that of radicals, can also inactivate DNA. The following observations suggest at least a partial answer to this question.

Several experiments indicate that the inactivation rate of DNA by HA is proportional to the concentration of produced peroxide (H_2O_2) as if that were in equilibrium with the concentration of the inactivating reagent. (1) Catalase and peroxidase prevent DNA

inactivation by HA at pH 7.5. (2) EDTA, pyrophosphate, cyanide, or $FeCl_3$ inhibit both peroxide production and DNA inactivation by HA. (3) In a 10^{-2} M HA solution of pH 9, both the peroxide concentration and the inactivating power increase for more than 1 hr.

Peroxides do not seem to inactivate DNA directly but *via* radicals. For example, inactivation of DNA by H_2O_2 is greatly enhanced by ultraviolet or Fe^{2+} (Butler and Smith, 1950; Butler and Conway, 1953; Moroson and Alexander, 1961), both of which are known to produce ·OH radicals from H_2O_2. The inactivation by H_2O_2 is furthermore inhibited by EDTA, pyrophosphate, cyanide, or Fe^{3+} (Freese and Gerson, 1965, unpublished data), just as observed for HA.

The presence of HA may not only be required to continuously produce new H_2O_2 but also to generate new ·OH radicals from H_2O_2 either directly by reaction 2 or indirectly, by reducing a trace metal (Me^{n+1} to Me^n) which can then be reoxidized by H_2O_2.

This ·OH generating mechanism would explain why a combination of HA and H_2O_2 had a much stronger inactivating effect than either of the agents alone (Figure 11): the added H_2O_2 could immediately produce OH radicals. Neither this combined inactivating effect nor the chemical reaction of H_2O_2 with HA were inhibited by EDTA, whereas the inactivating effect of each compound alone was strongly inhibited by EDTA. This suggests that the combined effect of HA + H_2O_2 was catalyzed by the metal-independent reaction 2, and that the concentration of radicals was preserved by the chain reaction 3 and 4. The combined inactivating effect was, however, inhibited by pyrophosphate or KCN, the same agents which also inhibited the destruction of H_2O_2 by HA (for explanation see above).

The conclusion that peroxide and thus radical production is required for DNA inactivation is further strengthened by results with HA derivatives. Two compounds, containing a free NOH group, have shown the same inactivating effect as HA: *N*-methyl-HA (CH_3-NHOH) (Freese and Freese, 1964) and *N*-hydroxyurethan ($C_2H_5OCNHOH$) (Freese, 1965). We have shown in this paper that the inactivating effect of *N*-methyl-HA is oxygen dependent and that peroxide is produced. The reaction with oxygen presumably is similar to (1)

$$H_3CNHOH + O_2 \rightarrow H_3CNO + H_2O_2 \qquad (5)$$

where the nitroso compound probably is immediately hydrolyzed to HNO and alcohol (Johnson *et al.*, 1956).

The HA derivative *O*-methyl-HA (NH_2OCH_3), in contrast, exhibited essentially only the mutagenic and not the inactivating effect (Freese and Freese, 1964). It did not produce H_2O_2. The free NOH group is therefore necessary for both the inactivating effect and peroxide production.

Altogether it seems clear that ·OH and other radicals are continuously produced by the reaction of hydroxylamines (with free NOH groups) with either oxygen or hydrogen peroxide and that these radicals can inactivate DNA. But since all these reactions probably also produce nitroxyl (HNO or :NOH), or the corresponding nitroso compound (5), it is not yet possible to decide whether only the radicals or also HNO are mainly responsible for the inactivating effect of HA.

Explanation of the HA Concentration Dependence of DNA Inactivation. The strange curve in Figure 6, which displays the dependence of DNA inactivation on the HA concentration, can now be explained, regardless of whether a radical (*e.g.*, OH) or nitroxyl is the inactivating reagent. In either case the production rate of R is $k_1[HA][O_2]$ and the destruction rate is the sum of k_2·[HA][R] (reaction of R with HA) and $k_3[D][R]$ (reaction with D = DNA). Since under the conditions of these experiments (pH 6.2 and 75°) the survivors decreased exponentially with time, the concentration of R must have stayed constant. We may therefore assume quasi-stationary state conditions in eq 6

$$k_1[HA][O_2] = k_2[HA][R] + k_3[D][R] \qquad (6)$$

and obtain eq 7.

$$[R] = \frac{k_1 Q}{k_2 + k_3[D]/[HA])} \qquad (7)$$

This formula describes quite well the observed HA and DNA concentration dependence of the inactivation rates. At HA concentrations $\leq 10^{-2}$ M one can assume that the reaction of oxygen with HA does not significantly change the oxygen concentration in the solution, *i.e.*, O_2 = constant. R is then independent of the HA concentration as long as the [DNA]/[HA] ratio is small enough that $k_2 \gg k_3[D]/[HA]$. For very small HA concentrations, however, the concentration of DNA becomes rate limiting and [R] decreases linearly with [HA], as we have observed (Figure 6). (One could determine the ratio k_2/k_3 from the intercept of the horizontal and the linear curve.) Equation 7 approximately describes also the competition between DNA and HA, shown in Figure 8. (The deviations from (7) may be caused at least partially by a change of the trace metal concentration, when different amounts of HA or DNA were added to the reaction mixture.)

The decrease of the inactivation rates at high HA concentrations can be explained by the concomitant decrease of the oxygen tension. Without HA, the oxygen concentration in water, exposed to an atmosphere of air, is about 2×10^{-4} M at 45° and 1.3×10^{-4} M at 70°. We have shown that this concentration decreases upon addition of HA (see Figure 12). The oxygen concentration in HA is determined by the rate (k_4) at which oxygen enters the solution from air, the rate ($k_5[O_2]$) at which it leaves the solution, and the rate ($k_1[HA][O_2]$) at which it is used up by the reaction with HA (k_4 and k_5 depend on the surface/volume ratio of the liquid). In a stationary state, one may therefore write

$$k_1[HA][O_2] + k_5[O_2] = k_4 \qquad (8)$$

or

$$[O_2] = \frac{k_4}{k_1[HA] + k_5} \qquad (9)$$

This value for O_2 can now be inserted into eq 2.

$$[R] = \frac{k_1 k_4}{\left(k_2 + k_3 \frac{[D]}{[HA]}\right)(k_1[HA] + k_5)} \quad (10)$$

For large HA concentrations R would then decrease

$$[R] = \frac{k_4}{k_1[HA]} \text{ for } k_1[HA] \gg k_1 \text{ and } k_2 \gg k_3 \frac{[D]}{[HA]} \quad (11)$$

in agreement with our observations.

The concentration of R may actually remain constant, at pH 6.2 and 75°, even for longer times than those used in inactivation studies because the rapid peroxide destruction by HA efficiently keeps the concentration of peroxide and thus R very low and constant. At pH 9, however, both the H_2O_2 concentration and the inactivation rate of later added DNA increase for more than an hour. This difference in the rate at which stationary conditions develop would also explain why the concentration of H_2O_2 produced by HA at pH 8.5 exhibited a bell-shaped curve (Figure 14), whereas the inactivation rates at pH 6.2 exhibited a trough-shaped curve. Kinetically, this difference would be reflected in a strong pH dependence of the rate constants k_i in (10).

When the HA concentration increased above 0.5 M the inactivation rate decreased more slowly or began to increase again. We have shown that this portion of the inactivation effect does not depend on the presence of oxygen and increases linearly with the HA concentration (see Figure 6). Such an increase has also been observed for the rate at which HA induces fluorescent mutants by direct action of HA on cytosine (see Figure 7) (Freese and Freese, 1964). The oxygen-independent lethal effect is therefore probably caused by the induction of lethal mutations in some vital genes that are included in the DNA pieces containing the tryptophan marker. The shape of the inactivation curve at high HA concentrations can thus be explained by the superposition of a linear decrease of the inactivating effect and a linear increase of the mutagenic effect with increasing HA concentration.

The above findings point out the ideal method for mutation induction by HA or its derivatives: One should employ high concentrations (≥ 1 M), at pH 6.2 or lower, in the presence of ≥ 0.05 M pyrophosphate.

Correlation between the Inactivating Effect and Chromosomal Breakage. HA and many of its derivatives (having free NOH groups) induce extensive chromosomal breakage and aberrations, *e.g.*, in hamster tissue cultures (Hsu and Somers, 1961; Somers and Hsu, 1962; Borenfreund *et al.*, 1964) and in *Vicia faba* (Kihlman, 1961). Many of these agents, particularly those which carry lipophilic groups, are also carcinogenic, *e.g.*, *N*-hydroxyurethan (Berenblum *et al.*, 1959), *N*-hydroxy-2-acetylaminofluorene (Miller *et al.*, 1961a), *N*-hydroxy-4-acetylaminobiphenyl (Miller *et al.*, 1961b), and *N*-hydroxyaminofluorene (Uehleke, 1963), or they have been used as antitumor agents (*e.g.*, hydroxyurea) (Stearns *et al.*, 1963). It seems likely that most of these effects are caused by the same oxygen-dependent reactions with chromosomal DNA which inactivate transforming DNA. If this were correct, it should be possible to show that oxygen tension, chelating agents, transition metals, or radical scavengers have a strong effect on the biological effect of these hydroxylamines.

Chromosomal breakage may come about in various ways. (a) We have shown (Freese *et al.*, 1965, unpublished data) that dilute concentrations of HA react with the DNA bases thymine and guanine, the reaction depending on oxygen. This base alteration may make the adjacent DNA backbone susceptible to spontaneous breaks, just as depurination does (Bayley *et al.*, 1961). (b) The altered DNA bases may also invite enzymes to break DNA further down, to repair the damage, or to initiate recombination. (c) The sugar phosphate backbone may be directly broken. Bendich *et al.* (1964) have observed a decrease of viscosity and sedimentation constant of isolated DNA in the presence of 0.1 M HA at neutral pH. Similar to our inactivating effect, this reaction was enhanced by oxygen and inhibited by EDTA. The authors proposed that DNA strands are broken by the specific actions of HA and hyponitrite on amino acid or peptide links. Our findings indicate that such a specific effect is not required to explain the data: on the one hand the reaction of the DNA bases loosens the DNA structure (which would already reduce the viscosity) and it may cause backbone breaks; on the other hand the produced radicals may directly break the sugar phosphate backbone.

Comparison with Other Agents. An oxygen dependence of inactivating and chromosome-breaking effects has also been found for ionizing radiations (Thoday and Read, 1947, 1949; for a review see Giles, 1952), 8-ethoxycaffeine (Kihlman, 1955), maleic hydrazide (Kihlman, 1956), cupferron (the ammonium salt of *N*-nitrosophenyl-HA), *N*-methylphenylnitrosamine (Kihlman, 1961), and 1-methyl-2-allylhydrazines (Berneis *et al.*, 1963). The last compounds have also been shown to produce H_2O_2, and ionizing radiations are well known to produce both radicals and peroxides.

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