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The Influence of High Oxygen Pressures on the Viscosity of Solutions of Sodium Desoxyribonucleic Acid and of Sodium Alginate[1]

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No change in the viscosity of 0.1% DNA or of 0.1% sodium alginate was detected when these solutions were exposed to high oxygen pressures for a period of 14 to 16 hours. When GSH was added to either DNA or sodium alginate and then exposed to high oxygen pressure, there was a definite decrease in viscosity due to the oxygen. Exposure of GSH solutions (unbuffered) to high oxygen pressure resulted in the oxidation of GSH and the formation of hydrogen peroxide. Both EDTA and thiourea inhibited the net formation of hydrogen peroxide by GSH solutions exposed to high oxygen pressure, and also inhibited the decrease in viscosity of DNA solutions (in presence of GSH) exposed to high oxygen pressure. Cysteamine, cysteine, ascorbic acid and sodium ascorbate also produced hydrogen peroxide in the presence of high oxygen pressure. Adding these substances to 0.1% sodium alginate solutions produced a definite decrease in viscosity, which was greatly augmented upon exposure to oxygen.

This investigation represents part of our effort to gain more information on the biological problem of oxygen toxicity. Gerschman and co-workers[2] have postulated that some of the biological effects of high oxygen tension and of the initial effects of X-irradiation may possibly be due to a common factor, presumably the formation of oxidizing free radicals. It is generally believed that ionizing radiation produces the free radicals H· and OH· in aqueous solutions.[3] Michaelis' theory of univalent oxidation,[4] implies the formation of free radicals as intermediates in oxidations. Not all oxidations would necessitate the formation of free radical intermediates,[5] but evidence has been given for the existence of free radicals in normal metabolism.[6] Such free radicals (R·) in theory could activate oxygen to oxidizing free radicals (RO_2·). In biological systems, both the reduction of oxygen and initial effects of X-irradiation might be mediated through the formation of oxidizing free radicals. It would be expected that an increased oxygen tension would accelerate the rate of formation of these oxidizing free radicals. Thus, in order to regulate oxidations the cell would be confronted with the necessity of controlling the potential destructive effects of oxidizing free radicals. If the oxygen tension is sufficiently increased, the cellular defenses would be overruled and so reveal the toxicity of oxygen.

Since high doses of X-rays have previously been shown by several workers[7-11] to cause a decrease in the viscosity of desoxyribonucleic acid (DNA), an effect attributed in great part to free radical action, it seemed of interest to investigate the effects of oxygen *per se* on the viscosity of DNA solutions[12] and of sodium alginate solutions, reported to exhibit a decrease in viscosity after X-irradiation.[13]

Method

Sodium desoxyribonucleic acid (DNA) was prepared by the method described by Kay, *et al.*[14] Solutions of 0.1% DNA were utilized in these experiments. The specific viscosities were determined in Ostwald-Cannon-Fenske viscosimeters using 6-ml. samples at 28.5°. The viscosities (V) were expressed as the percentage of the specific viscosity of the DNA solution exposed to room air.

The method for determining and expressing the sodium alginate (Kelcosol, Kelco Co.) viscosities was identical to the one described for DNA.

For the determination of hydrogen peroxide,[15] 0.2 ml. of a titanium sulfate solution (saturated titanium sulfate solution in 6 N H_2SO_4) was added to 5 ml. of test solution and the yellow color produced was quantitatively determined with a blue filter in a Klett-Summerson colorimeter. Centrifugation was necessary to eliminate a slight precipitate formed upon addition of the reagent to samples containing DNA. Solutions of hydrogen peroxide, standardized iodometrically, were used for calibrating the colorimeter.

The concentration of reduced glutathione (GSH) (Nutritional Biochemicals) was determined by iodometric titration using potassium iodate.[16] Because of the small amounts of hydrogen peroxide found in our experiments, it was necessary to multiply the concentration found by an approximate empirical correction factor which was equal to 1 + mM H_2O_2 present.

Other substances used in these investigations were: EDTA (ethylenediaminetetraacetic acid disodium salt) (ethylenedinitrilo)-tetraacetic acid disodium salt (Eastman Organic Chemicals), thiourea (Eastman Organic Chemicals), ethanol, cysteamine (mercaptoethylamine hydrochloride, Evans Chemicals), cysteine (L-cysteine hydrochloride, Nutritional Biochemicals), ascorbic acid (L-ascorbic acid, Eastman Organic Chemicals), sodium ascorbate (Nutritional Biochemicals), oxidized glutathione (GSSG) (Nutritional Biochemicals), and hydrogen peroxide (prepared from 30% H_2O_2, meets A.C.S. standards, Baker Chemical Co.).

The solutions were transferred into 15 ml. gauze-stoppered glass vials. For the 130 atmosphere studies, each vial was placed in a small stainless steel chamber and brought up to pressure. For the 6 atmosphere experiments, several vials were placed in a large pressure chamber and brought up to pressure after a thorough flushing of the chamber with the appropriate gas. The solutions were exposed to the gas for approximately 14 to 16 hours.

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Results

No effect on the viscosity of solutions containing only DNA could be detected after exposure to 130 atmospheres of either oxygen or nitrogen (Table I). The viscosity of DNA solutions in the presence of 3.26 m*M* GSH was decreased to about 37%; but if these solutions were exposed to high oxygen pressure (HOP), there was a further definite decrease in viscosity. Both EDTA and thiourea inhibited the effect of HOP on the viscosity of solutions of DNA containing GSH.

TABLE I

THE EFFECT OF HIGH OXYGEN PRESSURE ON THE VISCOSITY OF 0.1% DNA

Substance added	Gas	No. of determinations	V = [a] Viscosity
	130 atm. N_2	13	97.7 ± 2.3
.....	130 atm. O_2	18	98.5 ± 2.4
3.26 m*M* GSH	Room air	23	37.5 ± 3.6
3.26 m*M* GSH	130 atm. N_2	3	36.3 ± 3.4
3.26 m*M* GSH	6 atm. O_2	20	13.0 ± 1.4
3.26 m*M* GSH	130 atm. O_2	13	7.4 ± 1.5
3.26 m*M* GSH, 0.3 m*M* EDTA	Room air	3	60.3 ± 1.4
3.26 m*M* GSH, 0.3 m*M* EDTA	130 atm. O_2	5	56.8 ± 1.8
3.26 m*M* GSH, 3.26 m*M* thiourea	Room air	7	51.7 ± 2.4
3.26 m*M* GSH, 3.26 m*M* thiourea	6 atm. O_2	7	27.4 ± 4.6
3.26 m*M* GSH, 3.26 m*M* thiourea	130 atm. O_2	2	26.4 ± 10.4
3.26 m*M* GSH, 100 m*M* thiourea	Room air	8	53.8 ± 1.8
3.26 m*M* GSH, 100 m*M* thiourea	6 atm. O_2	8	50.4 ± 2.0

[a] Viscosity as expressed here is the % of the specific viscosity of the experimental solution divided by the specific viscosity of the DNA solution in room air (which was equal to 3.50 ± 0.12 in 35 determinations).

When 3.26 m*M* GSH (unbuffered solution, *p*H about 3.5) was exposed to 6 atomospheres of oxygen, there still remained 2.35 ± 0.11 m*M* (6 determinations) in the reduced state, but when exposed to 130 atmospheres, no reduced glutathione could be found (6 determinations). These findings were qualitatively confirmed by paper chromatography using phenol as the solvent in a descending system. It is of interest to mention that solutions of GSH exposed to room air were oxidized 2% per day.

During an experimental period of about 14 hours, no hydrogen peroxide was detectable in the GSH solutions exposed to room air or when water or pure DNA solutions were exposed to 130 atmospheres of oxygen. When 3.26 m*M* GSH was exposed to 6 atmospheres of oxygen, 0.20 m*M* H_2O_2 was found (Table II). At 130 atmospheres of oxygen, the net formation of H_2O_2 increased to 0.94 m*M*. Addition of 0.1% DNA to the GSH solutions resulted in a net decrease of H_2O_2 at both 6 and 130 atmospheres of oxygen. Both EDTA and thiourea also inhibited the net formation of H_2O_2 in GSH solutions (both in the presence or absence of 0.1% DNA exposed to HOP (Table II).

TABLE II

THE EFFECT OF HIGH OXYGEN PRESSURE ON THE PRODUCTION OF H_2O_2

Substance added[a]	O_2 pres. in atm.	No. of determinations	H_2O_2 m*M* found
3.26 m*M* GSH	6	29	0.20 ± 0.03
3.26 m*M* GSH	130	11	.94 ± .12
3.26 m*M* GSH + 0.1% DNA	6	21	.05 ± .01
3.26 m*M* GSH + 0.1% DNA	130	7	.45 ± .05
3.26 m*M* GSH + 0.3 m*M* EDTA	6	3	.00 ± .00
3.26 m*M* GSH + 0.3 m*M* EDTA	130	2	.00 ± .00
3.26 m*M* GSH + 0.3 m*M* EDTA + 0.1% DNA	6	3	.00 ± .00
3.26 m*M* GSH + 0.3 m*M* EDTA + 0.1% DNA	130	2	.00 ± .00
3.26 m*M* GSH + 3.26 m*M* thiourea	6	8	.07 ± .05
3.26 m*M* GSH + 3.26 m*M* thiourea	130	3	.06 ± .01
3.26 m*M* GSH + 3.26 m*M* thiourea + 0.1% DNA	6	8	.01 ± .01
3.26 m*M* GSH + 3.26 m*M* thiourea + 0.1% DNA	130	3	.00 ± .00
3.26 m*M* GSH + 100 m*M* thiourea	6	8	.03 ± .01
3.26 m*M* GSH + 100 m*M* thiourea + 0.1% DNA	6	8	.04 ± .01
3.26 m*M* cysteine	6	8	.23 ± .07[b]
3.26 m*M* cysteamine	6	6	.76 ± .02[b]
3.26 m*M* sodium ascorbate	6	6	1.07 ± .02[b]
3.26 m*M* ascorbic acid	6	8	1.33 ± .07[b]
1.07 *M* ethanol	6	2	0.12 ± .01

[a] GSH = reduced glutathione, DNA = desoxyribonucleic acid, EDTA = ethylenediaminetetraacetic acid disodium salt. [b] 12 determinations in room air for each of these 4 experiments gave values for H_2O_2 of 0.05 ± 0.00, 0.04 ± 0.01, 0.13 ± 0.01 and 0.32 ± 0.01, respectively.

Ethanol, cysteamine, cysteine, ascorbic acid and sodium ascorbate also produced H_2O_2 in the presence of HOP.

Table III shows that the effects of both oxygen and GSH on the viscosity of 0.1% sodium alginate solutions were similar to those observed on DNA solutions. The addition of oxidized glutathione (GSSG) produced a decrease in the viscosity of solutions of this polymer. However, solutions containing GSSG and exposed to oxygen showed no additional effect on the viscosity. Other substances which acted similarly to GSH on the viscosity of sodium alginate solutions in the presence or absence of oxygen were cysteine, cysteamine, ascorbic acid and sodium ascorbate.

On the other hand, the addition of hydrogen peroxide, itself, produced significant decreases in the viscosity of sodium alginate solutions.

Discussion

As an activator of oxygen, reduced glutathione was chosen because of its general biological importance and because it might act as a possible hydrogen donor. The necessity of using an activator became evident when no influence of HOP on the viscosities of DNA or sodium alginate was

TABLE III

THE EFFECT OF HIGH OXYGEN PRESSURE ON THE VISCOSITY OF 0.1% SODIUM ALGINATE

Substance added	Gas	No. of determinations	V = [a] Viscosity
	6 atm. O_2	14	98.3 ± 1.2
1 mM GSH	Room air	5	40.6 ± 3.6
1 mM GSH	6 atm. O_2	5	14.8 ± 1.5
3.26 mM GSH	Room air	10	26.7 ± 1.2
3.26 mM GSH	6 atm. O_2	9	8.8 ± 2.3
10 mM GSH	Room air	5	22.9 ± 0.6
10 mM GSH	6 atm. O_2	5	7.2 ± 0.7
0.5 mM GSSG	Room air	3	62.6 ± 1.1
0.5 mM GSSG	6 atm. O_2	3	63.9 ± 1.4
1.63 mM GSSG	Room air	3	51.7 ± 1.4
1.63 mM GSSG	6 atm. O_2	3	49.0 ± 1.6
0.326 mM cysteine	Room air	4	47.5 ± 4.6
0.326 mM cysteine	6 atm. O_2	4	9.5 ± 2.2
3.26 mM cysteine	Room air	5	15.0 ± 1.1
3.26 mM cysteine	6 atm. O_2	5	3.2 ± 0.5
3.26 mM cysteamine	Room air	4	31.4 ± 2.0
3.26 mM cysteamine	6 atm. O_2	4	9.7 ± 0.1
3.26 mM ascorbic acid	Room air	5	4.2 ± 0.5
3.26 mM ascorbic acid	6 atm. O_2	5	1.4 ± 0.4
3.26 mM sodium ascorbate	Room air	5	12.4 ± 0.6
3.26 mM sodium ascorbate	6 atm. O_2	5	3.0 ± 0.4
0.0163 mM H_2O_2	Room air	2	99.4 ± 4.8
0.05 mM H_2O_2	Room air	2	87.4 ± 4.8
0.163 mM H_2O_2	Room air	4	66.8 ± 5.0
0.5 mM H_2O_2	Room air	4	53.0 ± 6.7
1.63 mM H_2O_2	Room air	4	28.0 ± 4.3

[a] Viscosity as expressed here is the % of the specific viscosity of the experimental solution divided by the specific viscosity of the sodium alginate in room air (which was equal to 7.32 ± 0.07 in 83 determinations).

detected. Assuming an univalent oxidation of GSH, a simplified way to represent the reaction steps is given in Fig. 1. The oxygen would first be activated to the perhydroxyl radical ($HO_2°$) with subsequent formation of H_2O_2. The H_2O_2 so formed could then further oxidize GSH[17] as shown in eq. 5, probably also by a free radical mechanism.

FIGURE 1

GLUTATHIONE OXIDATION BY O_2

1. $GSH + O_2 \longrightarrow GS° + HO_2°$
2. $GSH + HO_2° \longrightarrow GS° + H_2O_2$
3. $2GS° \longrightarrow GSSG$

4. $2GSH + O_2 \longrightarrow GSSG + H_2O_2$
5. $2GSH + H_2O_2 \longrightarrow GSSG + 2H_2O$

6. $4GSH + O_2 \longrightarrow 2GSSG + 2H_2O$

or

$2GSH + {}^1/_2O_2 \longrightarrow GSSG + H_2O$

Equation 6 would represent the over-all reaction.[18] It has been reported that during the oxidation of GSH by molecular oxygen in the presence of a catalyst such as copper, small amounts of hydrogen peroxide were formed at a pH of 7.27[19] and at a pH range of 9 to 11.[20] From eq. 4 and 5, it could be expected that oxygen and hydrogen peroxide could compete with one another for the oxidation of glutathione. Yet at sufficiently high oxygen pressures, one might expect that the hydrogen peroxide produced could not react with the reduced glutathione, since the latter would be already in the oxidized state. Thus, a net formation of hydrogen peroxide would be evidenced. The maximum amount of hydrogen peroxide that could be formed would be obtained only if the reaction represented in equation 4 went to completion and no other occurred.

Although it is known that it becomes increasingly more difficult to oxidize GSH at a low pH,[21] we did observe significant oxidations of unbuffered GSH solutions upon exposure to 6 and 130 atmospheres of oxygen. At 6 atmospheres of oxygen, 2.35 mM of the original 3.26 mM GSH were still in the reduced state. This would mean that only 0.91 mM GSH actually was oxidized, which corresponds to the calculated maximum of 0.46 mM hydrogen peroxide (see eq. 4 from Fig. 1). Actually, the net concentration of hydrogen peroxide found was only 0.20 mM or 43% of the calculated maximum. At 130 atmospheres of oxygen, all the GSH was actually oxidized, corresponding to the calculated maximum production of 1.63 mM hydrogen peroxide, but actually only 0.94 mM hydrogen peroxide or 58% of the calculated maximum production was observed. This discrepancy might be explained by assuming that some GSH is oxidized by hydrogen peroxide instead of oxygen (see eq. 5 in Fig. 1). The possibilities of further oxidation states of GSSG or of hydrogen peroxide decomposition into water and oxygen cannot be disregarded entirely to account for part of this discrepancy.

When DNA was present in the GSH solutions, less hydrogen peroxide was detected at both 6 and 130 atmospheres of oxygen. If some precursor of hydrogen peroxide or hydrogen peroxide itself reacted with the DNA, this could account for the smaller amounts of peroxide detected. Although GSH decreased the viscosity of DNA and of sodium alginate, a still further decrease was observed when these solutions were exposed to HOP. Since GSSG had less of an effect than GSH on the viscosity of sodium alginate (Table III), the effect of HOP cannot be attributed to the GSSG produced. Since addition of hydrogen peroxide to the sodium alginate solutions (Table III) resulted in a decreased viscosity, at least part of the HOP effect must be due to hydrogen peroxide. However, the possibility still exists that some of the precursors of hydrogen peroxide, for instance the perhydroxyl radical and other free radicals contributed to the decrease in viscosity.

Inhibition by EDTA of GSH oxidation and of the decrease in viscosity of DNA solutions exposed to HOP in the presence of GSH might be explained by the removal of trace metal catalysts. In biological systems trace metal catalysts are present. From a theoretical point of view, however,

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it would be well to bear in mind that since catalysts can only change the rate of reaction and not the equilibrium states, the same results would be attained if enough time were allowed.

Thiourea has been shown to inhibit the decrease in DNA viscosity caused by X-irradiation and to accelerate the viscosity decrease produced by the presence of hydrogen peroxide.[9] It was therefore of interest to investigate the role of thiourea in our systems. It was found that thiourea inhibited the net production of hydrogen peroxide and also inhibited the decrease in DNA viscosity. The action of thiourea might be due to its role in decomposing hydrogen peroxide.[9,22]

Besides GSH, other substances which possess a labile hydrogen might be similarly expected in the presence of oxygen to form hydrogen peroxide and to produce a viscosity change of DNA and sodium alginate. Tables II and III show that cysteamine, cysteine, ascorbic acid and sodium ascorbate do possess such properties. For these substances, similar oxidation schemes to that of GSH can be postulated. Many oxidations of this type are catalyzed by metals, and these are reactions for which univalent shifts of electrons have been postulated.[4]

According to Mattill[23] "antioxidants" which inhibit oxidations by breaking reaction chains are characterized by the possession of a labile hydrogen. Substances such as ethanol, cysteamine, cysteine and glutathione, which may act as hydrogen donors, have been noted to protect mice against the toxicity of oxygen and of X-irradiation.[2] The formation of hydrogen peroxide in the presence of oxygen (Table II) can be taken as evidence that these substances do possess a labile hydrogen. Other investigators have also detected hydrogen peroxide in the oxidation of sulfhydryl compounds[24,25] and of ascorbic acid.[25,26] Yet, the possession of a labile hydrogen could also explain the ability of these substances to activate oxygen (see Fig. 1) and consequently act as pro-oxidants. The fact that antioxidants can act as pro-oxidants according to the characteristics of a system has been discussed by others.[23,27,28] Recently GSH has been reported to be toxic due to its pro-oxidative property.[29] GSH can increase the survival time of mice exposed to 6 atmospheres of oxygen,[2,30] but it decreased their survival in 1 atmosphere.[30] One interpretation of this study is that GSH activated oxygen and thus was a pro-oxidant. However, *in vivo* GSH might be preferentially oxidized instead of essential cell constituents thereby acting as a chain breaker. If so this would constitute an antioxidant action. Shelton and Cox[28] have recently discussed how substances can act as either antioxidants or pro-oxidants depending upon the circumstances. Other factors which should be taken into consideration in discussing antioxidant would be the removal of metal catalysts by chelating agents such as EDTA, and the inhibition of oxidation by inclusion.[31]

It is interesting to note that the viscosity decrease of nucleic acids by hydrogen peroxide is made more pronounced by adding "activating" substances such as ferrous ions, cysteine and ascorbic acid.[9,11,32,33]

In connection with the changes in viscosity of DNA, it is pertinent to mention that mutagenic effects produced by HOP have been produced in *E. coli*.[34]

In conclusion, our experimental results are not inconsistent with the idea that a free radical mechanism might be involved in the observed changes produced by oxygen *in vitro* as well as *in vivo*.

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