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Erythrocyte Survival in Sheep Exposed to Ozone

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Ozone has been reported to produce a variety of extrapulmonary effects including changes in the specific activity of some erythrocyte enzymes (BUCKLEY et al. 1975; GOLDSTEIN et al. 1968). BUCKLEY et al. (1975) reported statistically significant extrapulmonary changes in a number of human volunteers experimentally exposed to ozone at 0.5 ppm for 2.75 h. Included in the findings were increases in red cell osmotic fragility, serum vitamin E, and erthrocyte G-6-PD activity while decreases occurred in acetylcholinesterase (ACHase) activity and glutathione. HACKNEY et al. (1977) exposed human male volunteers to 0.37 ppm of O_3 for 2.0 h and reported similar effects including increases in seum vitamin E and G-6-PD activity while ACHase activity decreased. The G6PD increases in the anucleate red cell population are thought to represent a decrease in mean red cell age consistent with reticulocytosis and a rather significant acute hemolysis (NAS 1977). This interpretation is supported by MUDD & FREEMAN (1977) and FREEMAN et al. (1979) who suggest that older cells are more susceptible to oxidant damage. Such cells would more likely become sphered (LAMBERTS et al. 1964) resulting in an increased rate of splenic sequestration of the misshapen or fragile cells (WALLS et al. 1976). The older and more susceptible cells would be selectively filtered out of the systemic circulation effectively resulting in a lowering of the mean age of the remaining cells. Younger cells are known to have higher ATP levels, are less osmotically fragile (COHEN et al. 1976), and have greater G6PD activity (TEIGE et al. 1976). Therefore, it is attractive to postulate that exposure to O_3 causes a more rapid "aging" of the erythrocytes that are subsequently sequestered by the spleen. If sequestered cells are the older cells, those remaining in the circulation would be "younger" and possess higher enzymatic activity. However, there is no published evidence to indicate that a marked hemolysis occurs following O_3 exposure (NAS 1977) and tentative data indicating an increased splenic sequestering of erythrocytes following acute O_3 exposure is unpublished (BUCKLEY et al. 1977). It was the purpose of this study to evaluate the hypothesis that ozone exposure produces decreased cell survival which may be the result of premature erythrocyte aging. In order to evaluate this hypothesis, erythrocyte survival studies using Cr_{51} were performed. The animal model selected for this study was the Dorset ewe. The adult has a body weight comparable to humans (40-60 kg) making techniques for studying erythrocyte survival more easily applied. Further, this strain of sheep has an

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an erythrocyte G-6-PD activity that is very low, being comparable to human A-variants with G6PD deficiency. Such a deficiency has been associated with acute hemolytic episodes from oxidant chemicals (BEUTLER 1972) and it has been postulated that O_3 exposure may produce hemolytic effects in G6PD deficients more readily than in persons with normal activity (CALABRESE et al. 1977). Therefore, an animal model with a G6PD deficiency should experience a marked decrease in erythrocyte survival from O_3 exposure when compared with other animal models. Dorset sheep were then considered to be the most appropriate animal model for evaluating this hypothesis.

METHODS

Animal Exposure. Fifteen Dorset ewes were obtained from the University of Massachusetts campus and fed ad libitum water, hay, and a salt lick, along with approximately 4-8 ounces of dried ground corn meal each day. Each sheep served as its own control, and since it was imperative that all events in the control exposure using filtered room air resemble those in the O_3 exposure, the presence of O_3 in the exposure chamber was the only measured difference in experimental conditions that existed between the control exposure and the O_3 exposure. There was a single 2.75 h exposure at 0.25, 0.50, and 0.70 ppm.

The stainless steel, air tight exposure chamber was equipped with a built-in plate glass viewing window, and has inside dimensions approximately 3 feet wide x 5 feet high x 5 feet deep, yielding roughly 75 cubic feet of air space and was constructed by Young and Bertke Company of Cincinnati, Ohio. A metabolic-type restrainer/dolly was employed to secure the sheep within the chamber. An intake and exhaust plenum acted to maintain an even current of filtered air within the chamber at a flow of 15 SCFM. The sheep were randomly selected for the sequence of exposure through the use of a random numbers table of the Rand Corporation. After the exposure order was determined, the random numbers table was employed to delegate five sheep to each of the three O_3 concentrations under study.

CR-51 Labeling. Each ewe received a total of two red cell taggings, one before the control exposure and one before the O_3 exposure. The quantity administered at each labeling was approximately 120 microCuries (μCi) per sheep. A syringe was used to withdraw 20 mL of blood from the jugular vein of each sheep. The syringe was prewetted with acid-citrate-dextrose (ACD) modified anticoagulant solution to prevent clotting during the collection. The blood sample was then transferred to a sterile 50-mL rubber-capped serum vial, containing 4 mL ACD solution obtained from Mallinckrodt Nuclear of St. Louis, Missouri. A 1:5 mixture of ACD solution to blood was employed, since the survival of labeled cells may be reduced if they are incubated with an excess of ACD solution (POWSNER & RAESIDE 1971). Best results have been obtained by combining the whole blood sample with the ACD solution before the Cr-51 is added.

The chromium isotope was obtained from New England Nuclear of Boston, MA in the form of sodium chromate and was diluted with normal saline to a concentration of 150 μCi/mL and sterilized before being shipped.

The volume of Cr-51 introduced to the ACD-blood mixture was corrected to account for the physical decay of the isotope on each labeling day to yield a quantity of 150 μCi. The ACD-blood-isotope solution was incubated at room temperature (25°C) for 1 h with frequent gentle mixing. The initial concentration of 150 μCi/mL was high enough to prevent excessive dilution of the cells in the final mixture, while the complete mixing prevented a localized, excessively high chromium concentration with possible damage to surrounding cells. Chromium reversibly attaches to the cell membrane quite rapidly, however the nearly irreversible binding to the cell hemoglobin requires a minimum of 30 min (POWSNER & RAESIDE 1971). After the incubation period, 20 mL of the blood mixture was returned to the sheep's circulation by injection into the jugular vein.

Blood Collection. The initial blood sample was collected from the jugular vein opposite the injection site at time 0 (24 h postinjection). This time span allowed virtually all the chromium in the plasma to be excreted, so that no Cr-51 plasma correction was necessary. Since blood sampling, in itself, is capable of lowering the level of radioactivity in the circulation, the volume collected was restricted to 4 mL on alternating days. Vacuum tubes containing dry EDTA anticoagulant were used so that the total red cell volume was not affected (POWSNER & RAESIDE 1971). Blood collections opposite the injection site preclude obtaining erroneously high counts due to any chromium infiltration of tissues during the labeling process.

Chamber Exposure. The sheep were secured in the restrainer/dolly immediately prior to the collection of the time 0 blood specimen. After the blood was obtained, nonreactive vinyl endotracheal tubes were inserted into each nostril of the ewe in an attempt to deliver an optimum concentration of O_3 to the lungs. This latter action is prompted by research that indicated the high efficiency of the nasopharyngeal passage in removing O_3 from the airstream (HAZUCHA et al. 1973; MILLER 1979). It was found that this efficiency is greatest under low flow and low concentration conditions (YOKOYAMA & FRANK 1972; STOKINGER 1965). Also, mucus has been found to reduce O_3 as it passes through the airways (HAZUCHA et al. 1973). Therefore, if O_3 is to produce any changes in the lungs and blood, it must contact these parts in a sufficient concentration for a sufficient length of time (STOKINGER 1957). Since this study employed low O_3 concentrations, the amount of surface area of the respiratory tract constituted a major determinant of the O_3 concentration reaching the lungs. By shortening the reactive portion of the airway, it was anticipated that non-reactive tubes would reduce that amount of O_3 that might normally have been absorbed on nasopharyngeal membranes prior to entry into the lung tissues. During each exposure, the chamber

temperature and ambient humidity levels were monitoried. A Taylor sling psychrometer was used to obtain the relative humidity values.

Ozone Generation. The O_3 used in this study was generated on site by a Welsbach Laboratory Ozonator Mode T-408, a product of Welsbach Ozone Systems of Philadelphia, PA. The ozonator is an apparatus that passes dry air through a silent high-voltage electric discharge. Zero air (air especially free of water vapor, nitrous oxide, nitrogen dioxide, sulfur dioxide, and ozone) was supplied to the ozonator by the Model 110 Zero Air Supply, manufactured by the Thermo Electron Corporation of Hopkinton, MA. The O_3 concentration in the chamber was monitored by the Dasibi Model 1003 AH Ozone Analyzer, a product of Dasibi Environmental Corporation of Glendale, CA. This instrument measures gaseous ozone by means of ultraviolet absorption. Non-reactive teflon tubing was used to make all necessary connections.

A strip chart recorder was utilized to monitor the O_3 levels. These recordings were used to calculate an average concentration of O_3 during the timed exposure.

A sample of blood was removed from each 4 ml blood collection tube for a microhematocrit determination immediately after collection. An IEC Model MB Microhematocrit Centrifuge with a rated operation of 11,500 rpm was used to obtain the packed cell volume. A microhematocrit tube reader yielded the % hematocrit values, after the 3.5 min centrifugation.

A 1 mL sample of blood was placed in a plastic, capped tube for subsequent radioactive counting. The plastic, capped blood vials were stored at 4°C and accumulated so that all samples were counted on the same day. This eliminated the need to correct for the physical decay of the isotope (POWSNER & RAESIDE 1971). The samples were counted in a Beckman Gamma 4000 gamma counter, produced by Beckman of Irvine, CA. This instrument is equipped with a well-type sodium iodide (NaI) scintillation crystal. Adjustments were made in order to optimize its counting efficiency of the 0.322 MeV Cr-51 photon while screening out as much natural background radiation as possible.

A preinjection blood sample was counted along with the survival samples to allow measurement of background radiation and any inherent blood radioactivity. This value was substracted from the gross count rate of each sample to yield a net count rate per mL of whole blood.

Calculation of Red Blood Cell Survival. The calculations were based upon the 24-h sample (time 0) as being 100%. All subsequent samples were expressed as a percent of the 24-h sample, and indicated the percent radioactivity remaining in the circulation. If any samples indicated hematocrits different from the 24-h sample, the following correction was made:

$$\% \text{ Remaining} = \frac{\text{net whole blood count (each sample)}}{\text{net whole blood count (at 24 hours)}} \times \frac{\text{hematocrit (at 24 hours)}}{\text{hematocrit (each sample)}} \times 100$$

The % remaining activity was plotted on the logarithm scale on semi-logarithm paper versus time (in days) along the linear axis. A line was drawn through the points and then extended back to time 0. The apparent half-life is the time required for half of the radioactivity to leave the blood (POWSNER & RAESIDE 1971). Therefore, the red blood cell survival time was obtained by noting the number of days required for the % remaining to reach 50%.

Since each animal served as its own control, the statistical significance of any differences between the filtered air control and O_3 exposure survival rates were analyzed with the paired t-test with the critical level at $p \leq 0.05$.

RESULTS

Mean hematocrit values were determined for each sheep based on eight blood samples (Table 1). In all cases, the exposure mean hematocrit was less than the control mean hematocrit.

TABLE 1

Exposure Groups (ppm Ozone)	Control Mean Hematocrit	Exposure Mean Hematocrit	Statistically Significant Diff. (p)
0.25	33.5 ≠ 2.15	30.8 ≠ 2.9	p = 0.10
0.50	32.9 ≠ 3.44	30.9 ≠ 3.3	p = 0.10
0.70	33.0 ≠ 1.3	31.4 ≠ 2.0	p = 0.01

A two way analysis of variance (ANOVA) was performed on the control and exposure hematocrit values to detect any effects due to the various exposure levels, the ozone treatment, and/or any interaction between the exposure levels and treatment. The hematocrit values were not found to differ significantly between the three exposure levels. However, the exposure hematocrits were found to be significantly lower than the control hematocrits ($p = 0.005$). These results were performed on HCT's measured over the entire test period. When HCT's were compared between the exposure day (day '0') and the final days of the experiment (13-18 days post exposure), the results in Table 2 were obtained. There was a reduction in HCT values that occurred even for controls. When sheep were handled identically to exposure conditions but with 0.0 ppm of O_3 flowing through the chamber, the HCT's decreased from the '0' exposure day to the final day of measurement by 2.9, 1.9, and 2.4 percent. Test animals also showed decreases from initial to final measurements of

HCT, but consistently showed larger decreases in HCT than controls. The 0.25 ppm exposure group showed a net greater decrease in HCT of 0.8% when compared with its control. Differences for the 0.50 and 0.70 ppm groups were 0.6% and 0.3% respectively. Therefore, the largest net decrease in hematocrit occurred in the 0.25 ppm exposure group while the smallest decreases occurred in the 0.70 ppm exposure group thus suggesting an inverse relation between exposure level and effect.

TABLE 2

Net Changes in Hematocrit from Initial Measurement ('0' days exposure) to Final Measurement (13-18 days post exposure) for Control (0.0 ppm O_3) and Test Sheep Exposed to 0.25, 0.50, and 0.70 ppm O_3

	LEVEL OF EXPOSURE		
	0.0 ppm	0.0 ppm	0.0 ppm
Control (Initial-Final HCT)	2.9%	1.9%	2.4%
	0.25 ppm	0.50 ppm	0.70 ppm
Test (Initial-Final HCT)	3.7%	2.5%	2.7%
Net Difference (Control-Test HCT)	-0.8%	-0.6%	-0.3%

Figures 1-3 are composite representations of average percent remaining Cr-51 activity through 12 days in erythrocytes of five Dorset ewes after exposure to 0.25 ppm, 0.50 ppm and 0.70 ppm ozone, respectively, for 2.75 hours. Paired t-tests utilizing each mean difference and comparing it to zero (i.e., no significant difference between the control and exposure groups in the number of days elapsed to reach 75%, 50%, and 40% remaining activity) were performed. The results of the paired t-tests indicated no significant differences between the control and exposure groups in the time elapsed for each to reach 75% remaining activity.

When the mean differences in days between the control and exposure groups for 50% of the remaining Cr-51 activity in labeled erythrocytes were compared, the 0.25 ppm group was found to have a significant mean difference. The mean time elapsed for blood levels to reach 50% activity was 1.06 days sooner for the exposure group than for the control group. The 0.50 ppm and 0.70 ppm groups did not indicate any significant differences between the control and

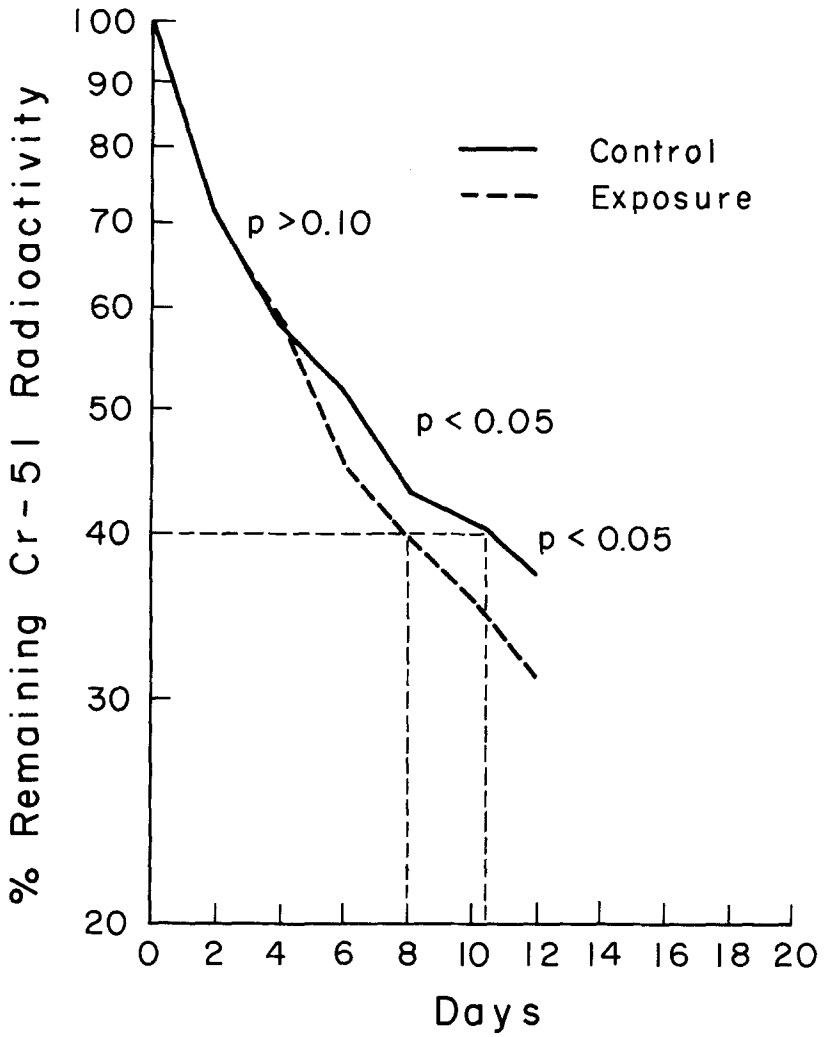

Figure 1. Average Percent Remaining Cr-51 Activity Through 12 Days in Erythrocytes of Five Dorset Ewes after Exposure to 0.25 ppm O_3 for 2.75 Hours.

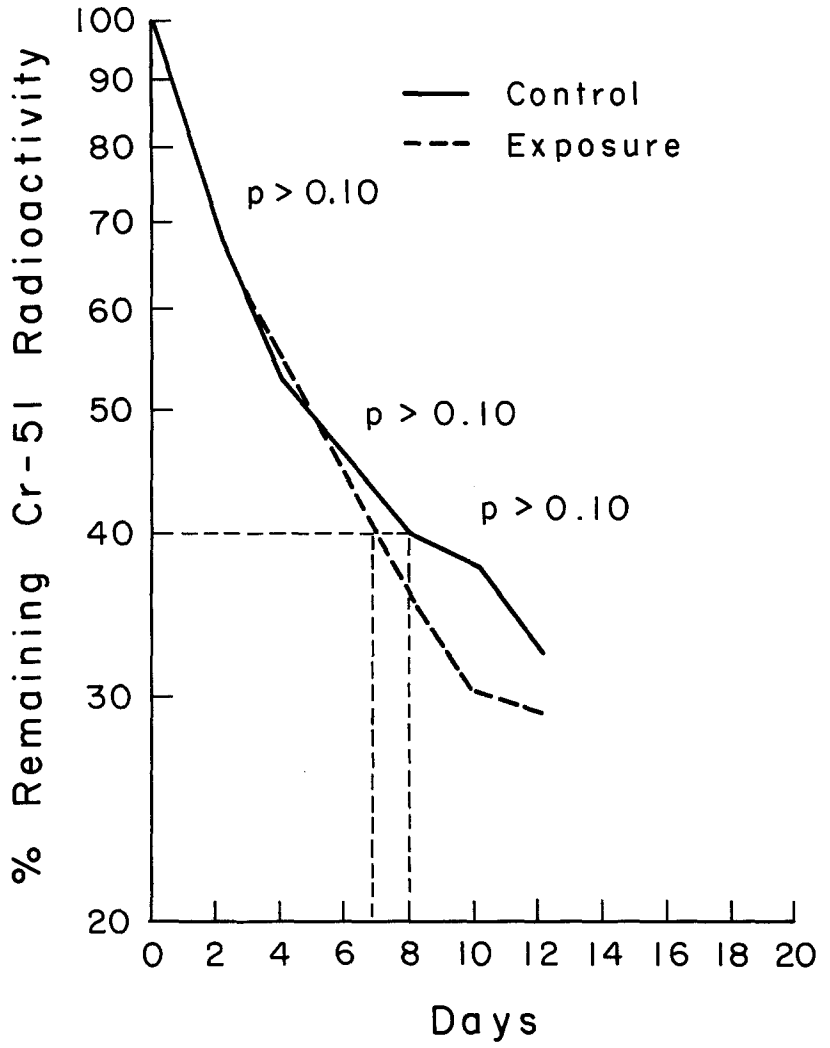

Figure 2. Average Percent Remaining Cr-51 Activity Through 12 Days in Erythrocytes of Five Dorset Ewes after Exposure to 0.50 ppm O_3 for 2.75 Hours.

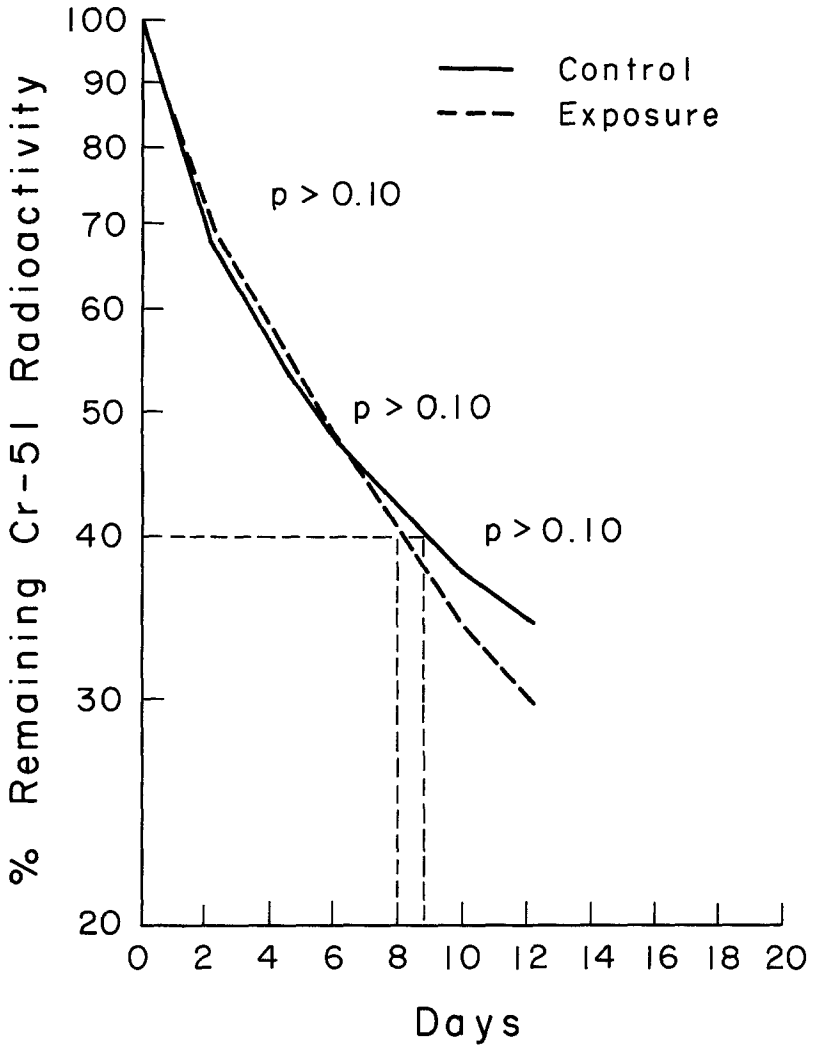

Figure 3. Average Percent Remaining Cr-51 Activity Through 12 Days in Erythrocytes of Five Dorset Ewes after Exposure to 0.70 ppm O_3 for 2.75 Hours.

exposure groups.

The mean differences between the control and exposure groups for 40% of the remaining Cr-51 activity in labeled erythrocytes were compared and the 0.25 ppm exposed groups reached 40% remaining activity an average of 2.54 days sooner than the control group. The 0.50 ppm and 0.70 ppm groups did not indicate any significant differences between the control and exposure groups in the amount of time elapsed to reach 40% remaining activity, although the mean survival times of erythrocytes appeared consistently less for the exposed sheep than for the controls.

DISCUSSION

The unanimous decrease in hematocrit values observed in the ozone exposed groups may be attributable to the inhalation of the oxidant. However, several factors are known to adversely affect hematocrit levels. For instance, elevations of serum electrolytes may cause a decrease in hematocrit values. Subnormal values may also be produced by an increase in plasma volume (LEAVELL & THORUP 1976). Likewise, hematocrits may fall when there is an underproduction of red blood cells, and excessive destruction of red cells or a significant blood loss (SIMMONS 1973). Ozone may be influencing one or more of these factors to produce a decrease in the hematocrit, but presumably it is the destruction of red cells that produces the decreased HCT as evidenced by the decreased erythrocyte survival.

Ozone has been observed to induce a sphering of red blood cells which could result in an increased rate of splenic sequestration. This selective filtration in the spleen removes susceptible cells from the circulation (FREEMAN et al. 1979). If the rate of splenic sequestration exceeds red cell production, a decreased hematocrit may result.

A significant finding regarding the effect of ozone upon red blood cell survival relates to the time frame within which the oxidant exerts its effects. With respect to red cell destruction, ozone does not appear to act immediately, but rather there appears to be a delayed effect. The susceptible cells may be damaged rather quickly upon contact, but this damage does not appear to be severe enough to force the cells immediate removal from the circulation. Nonetheless, these cells may be marked for destruction which would provide for the observed delayed effects.

This reasoning is substantiated at the 75% remaining activity level which reveals no significant differences between the control and exposed groups regarding the decline in red cell radioactivity, i.e., decline in labeled red cells presumably due to hemolysis. The cells may be marked for destruction at this point, but they are still capable of remaining in the circulation. The absence of an exposure level effect coincides with the delayed action proposed.

More time has passed at the 50% remaining activity level and a

significant difference between the control and exposed group is apparent. At the 0.25 ppm level, the exposed group reached the 50% remaining level an average of one day sooner than the control group, i.e., the blood radioactivity level dropped an average of one day sooner presumably due to increased red cell destruction.

The absence of significant differences between the control and exposed groups at the 0.50 ppm and 0.70 ppm groups suggests and inverse dose response relationship. Further, when net changes in hematocrits were compared from initial measurement ('0' days exposure) to final measurement (13-18 days post exposure) for control (0.0 ppm O_3) and test sheep exposed to 0.25, 0.50, and 0.70 ppm O_3, the results indicated a net decrease in hematocrit that was greater for the 0.25 ppm O_3 exposure group than for any of the other exposure levels. This indicated an inverse relation of exposure to effect as well.

The reason why ozone appears to exert a more deleterious effect on the 0.25 ppm group than on the 0.70 ppm exposure group may be related to ozone's property of being an irritant. The respiratory tract may react to this irritant through the production of excess mucus. Since mucus has been reported to reduce ozone as it passes through the airways (HAZUCHA et al. 1973), exposure to the 0.50 ppm and 0.70 ppm concentrations may have represented ozone levels high enough to induce this physiologic response. Such a release of mucus may have served to decrease the effective concentration of ozone reaching the lungs, passing through the air/blood barrier and consequently stressing the red cells. The 0.25 ppm ozone concentration may have minimally elicited this mucus response such that higher concentrations of ozone actually contacted the red cells. Additionally, ozone exposures of 0.25 ppm and greater have been found to produce edema in rats during or shortly following exposure (ALPERT et al. 1971). SCHEEL et al. (1959) reported that rats exposed to ozone showed immediate decreases in minute ventilation, tidal volume, and oxygen uptake which they associated with pulmonary edema. Ozone has also been found to constrict the lumen of the bronchi (HAZUCHA et al. 1973). This narrowing of the breathing passages may result in the reduced quantities of air reaching the lungs per unit time: the higher ozone concentrations may induce increased mucous protection, edema, and bronchoconstriction which reduce the effective ozone level reaching the lungs and blood, while the 0.25 ppm level failed to fully activate this mechanism. This proposed concept would allow for potentially higher ozone levels stressing the red cells in the 0.25 ppm group than at the higher levels.

The results at the 40% remaining Cr-51 activity level mirror those at the 50% remaining level, with the exception that no statistically significant exposure level effect could be demonstrated. Although the rate of cell destruction is not significantly higher for the 0.25 ppm level, the trend towards an inverse dose-response relationship between ozone exposure level and red cell destruction can still be inferred due to the lack of a demonstrable relationship.

The uniqueness of this study lies in the fact that is the first reported case depicting the effects of the oxidant ozone on red blood cell survival. In addition, the observation that ozone may not act immediately but exerts a delayed action effect marking red cells for future destruction also represents a unique finding, although the phenomenon of ozone producing delayed effects has been cited with other cells. MERZ et al. (1975) studied the circulating lymphocytes of humans experimentally exposed to ozone at 0.5 ppm for 6-10 hours. A statistically significant increase in the number of minor chromosomal abnormalities was observed that reached a peak two weeks after exposure before returning to normal. This delay in the development of chromosomal abnormalities following ozone exposure has been seen in hamsters and humans. In order to further evaluate the effects of ozone on the red blood cells of animals or humans, future research should study the effects of varied lengths of O_3 exposure on red blood cells in order to evaluate the presence of a delayed action effect.

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