CYTOTOXICITY AND SPECTROSCOPIC INVESTIGATIONS OF ORGANIC FREE RADICALS IN FRESH AND STALE COAL DUST

N.S. DALAL* • B. Jafari* • V. Vallyathan† • F.Y.H. Green†

*Chemistry Department, West Virginia University, Morgantown, WV 26506, USA
†Department of Pathology, West Virginia University, Morgantown, WV 26506, USA

INTRODUCTION

The mechanistic details of the biological events leading to coal workers pneumoconiosis (CWP) are not yet fully understood, despite several decades of extensive epidemiologic[1,2] and laboratory studies.[2-6] Epidemiologic studies[1,2] have shown, for example, that the incidence and severity of CWP differ markedly in the different regions and mines at comparable exposures, but laboratory investigations[2-6] have demonstrated only partial correlation of the epidemiologic data with the differences in the mineral composition and the rank of coal mined. In particular, while epidemiologic data indicate[1,2] direct correlation of the prevalence and severity of CWP with the rank (i.e., % carbon content) of coal, this correlation has not been established by laboratory[4-6] and animal exposure[3] studies. In order to explain these results, in 1980 Artemov and Reznik[7] suggested that perhaps they arise from the fundamental differences in the surface properties of the coal dusts inhaled by the coal miners and those used in the laboratory studies: while the miners inhale freshly fractured coal particles, henceforth called the 'fresh' coal dust, the laboratory studies generally utilize 'aged' coal dusts (i.e., dusts that have been stored for many days or even longer). Reznik and Artemov[7] used electron spin resonance (ESR) spectroscopy to show that the mechanical crushing of some Soviet Union coals generated organic free radicals and that the concentration and the decay times (hence the reactivity) of these radicals were higher for the coals of higher ranks. Since some of the radicals decayed within a few minutes in air, the authors surmised that while these radical species could lead to certain specific pathogenic reactions at the sites of mining operations, this might not be the case in the laboratory studies, due to the conventional use of 'stored' dusts which would be expected to contain significantly smaller free radical concentrations. This is consistent with the recent findings that, standardized, aged coal dusts exhibit minimal cytotoxicity.[8]

While the above mentioned work of Artemov and Reznik[7] did suggest a possible new clue to the pathogenesis of CWP (i.e., the role of the coal based free radical species), no direct biological/cytotoxicity data were provided to show that 'fresh' coal dusts were, indeed, more pathogenic than the 'stale' ones. Because of the rather significant implications of the Artemov-Reznik hypothesis to the understanding of the biochemical mechanism and, hence, the strategies for the eventual containment of CWP, we have initiated a comparative study of the free radical formation and cytotoxicity properties of freshly crushed coal particles. As done by Artemov and Reznik,[7] we have used the ESR technique as the direct method of measuring the concentration and decay kinetics of the coal based free radicals. Our preliminary ESR studies on two Pennsylvania coals, a bituminous (carbon content 72%) and an anthracite (95% carbon) coal, have confirmed the Artemov-Reznik finding that the crushing-induced free radical sites are higher on the coals with higher carbon content (i.e., higher rank).[9-11] In the present work we describe our more recent results of a parallel study of the time dependence of the decrease in the free radical content of a freshly made anthracite dust (as measured via ESR) and that of the dust's cytotoxicity potential as measured by the extent of hemolysis of (sheep) erythrocytes. We have also investigated the effects of free radical scavengers on the cytotoxicity potential of the dust and deduce that indeed the free radicals could play a significant role in the initial events in the mechanism of the cytotoxic effects due to the inhalation of coal dust inhalation.

MATERIALS AND METHODS

Reagents

The anthracite coal (#PSOC-867 Carbon content = 95%) was obtained from the Generic Respirable Dust Technology Center, Pennsylvania State University, University Park, Pennsylvania, USA. The samples were received as particles of about 5 mm (longest) dimension. These particles were hand crushed using an agate mortar-pestle arrangement to sizes smaller than 20 microns. A mixed particle size, rather than a specific size fraction, was used in our studies as an effort to simulate the rather random, respirable size coal dust particles in the mining operations. Superoxide Dismutase (SOD) and catalase were purchased from Sigma and were used as received.

ESR Measurements

ESR spectroscopy was used for identifying the crushing-induced coal radicals, and to follow their concentration. The ESR measurements were made with a Bruker ER-200D spectrometer operating at X-band (≈9.5 GHz) frequencies, with

100 kHz field modulation. The magnetic field is controlled via a linearized Hall probe (Bruker, model ER031M) and calibrated with a self-tracking NMR gaussmeter (Bruker, model ER035M). The microwave frequency was measured with a Hewlett-Packard 5340A digital frequency counter. All ESR measurements were carried out at room temperature.

Hemolysis Measurements

Hemolytic activity of the coal dust was measured, following an established method,[12] as the amount of hemoglobin released from a 4% suspension of sheep erythrocytes after incubation with 5 mg and 10 mg of coal dust for one hour at 37°C. The hemoglobin release was estimated via the absorbance at 540 nm using a Gioford spectrophotometer. The procedure was phosphate buffer solution as a negative control (background) and 0.5% Triton-X-100 as a positive control (100% hemolysis). The percentage of hemolysis was calculated as follows:

$$\% \text{ Hemolysis} = (I_{coal} - I_{neg}) / (I_{pos} - I_{neg})$$

where I_{coal} is the absorbance after incubation with the silica dust, while I_{neg} and I_{pos} are those with buffer only and 0.5% Triton-X-100, respectively.

RESULTS AND DISCUSSION

Radical Concentration vs. Crushing Under Nitrogen

Figure 1 shows three typical, first derivative, ESR spectra of the Pennsylvania anthracite coal (PSOC-867). Figure 1 (a) corresponds to the radicals from the stale, uncrushed particles while (b) and (c) are the spectra from 200 x 200 mesh (smaller than 40 micron) and the 400 x 400 mesh (smaller than 20 microns) particles. All of the signals are assigned to the highly delocalized carbon-centered organic free radicals, based on the measured g-value of 2.0029, Lorentzian lineshapes with peak-to-peak widths of about 1 Gauss.[13] Since the lineshapes and widths of all three spectra are essentially the same, the peak-to-peak heights of the first derivative spectra are proportional to the radical concentration in the respective preparations. It is evident that the smaller the particle size the larger the free radical concentration. The measured radical concentrations for all three samples are present in Table I.

Radical Decay in Air

As noted earlier,[9,13] the free radical signals decreased upon exposure of the samples to air, or oxygen. In order to investigate the effect of the crushing in air, as would be the case in the mining environment, some particles (the more shiny ones) were crushed in air to sizes of smaller than 25 microns and kept in air contact during ESR measurements every five minutes over 170 hours, without disturbing the sample or the spectrometer settings. Figure 2 shows three typical ESR spectra taken dependence of the free radical concentration. We note here that the radical concentration was measured from the areas under the ESR signal via double integration of the derivative peaks. It is seen from Figure 2 that the radical decay pattern seems to exhibit an oscillatory behavior up to about 24 hours after which the decay is monotonous. While much more detailed experimentation is necessary to establish the origin of this complex decay kinetics, it was found reproducible in two independent sets of measurements. Table II lists some of the selected data points. While the mechanism for the complex decay kinetics is not clear, a somewhat similar fluctuational behavior was noted in the hemolysis measurements as discussed next.

Hemolysis By Fresh vs. Stale Coal Dust

In order to determine if the freshly-crushed coal particles, containing higher amounts of free radicals, are more cytotoxic than the same particles on storage, we carried out hemolysis measurements on dust particles from the same stock as used in the above discussed radical kinetics. The hemolysis measurements were made for two dust concentrations at specific times (0–1/2, 4, 24, and 96 hours) after crushing. The average hemolytic activity was determined as 24.5% for the 5 mg/ml and 45.3% for the 10 mg/ml coal dust samples for the 0–1/2 hour period. The other measured values are included in Table III and represented graphically in Figure 3. Both Table III and Figure 3 reveal that the hemolytic activity decreased significantly as a function of the dusts' storage in both air and in a phosphate buffered saline (PBS) solution. It is also seen that the air stored dust samples exhibited a faster decrease in the hemolytic activity as compared to the PBS-stored samples.

Effect of Radical Scavengers

The above ESR measurements on 'fresh' coal dust demonstrated that the free radical sites on the coal particles react with oxygen in the air, and that this reaction increases the cytotoxicity of the dust particles. In order to find further clues to the biochemical mechanism of the oxygen-radical involvement in the cytotoxicity, hemolysis measurements were made in the presence of several oxygen-radical scavengers as discussed below.

Superoxide Dismutase (SOD)

The superoxide dismutase (SOD) was the first radical quencher enzyme tested for its effect on the hemolysis because SOD is known to provide an enzymatic defense mechanism against oxygen toxicity.[14] Thus if oxygenated radicals contribute to the toxicity of the fresh coal dust as measured via hemolysis, the addition of SOD should cause a decrease in the hemolysis. Table IV shows the results of addition of 0.5 mg/ml of SOD to the fresh coal dust samples prior to incubation with the sheep erythrocytes. As before, the hemolysis measurements were made at the dust concentrations of 5 mg/ml and 10 mg/ml. Indeed the addition of SOD causes a significant drop in the hemolytic potential of the fresh coal dust. The results indicate a significant role of the superoxide-based radicals in the hemolysis by coal dust.

Catalase

As an aid in understanding the above results with SOD, we investigated the effect of catalase, an enzyme known to offer protection against the hydrogen peroxide (H_2O_2) toxicity, by breaking down H_2O_2 into H_2O and O_2. Table IV also includes the hemolysis results in the presence of 0.5 mg/ml of added catalase. It is clearly seen that the addition of catalase decreases the hemolytic activity even more than done by SOD.

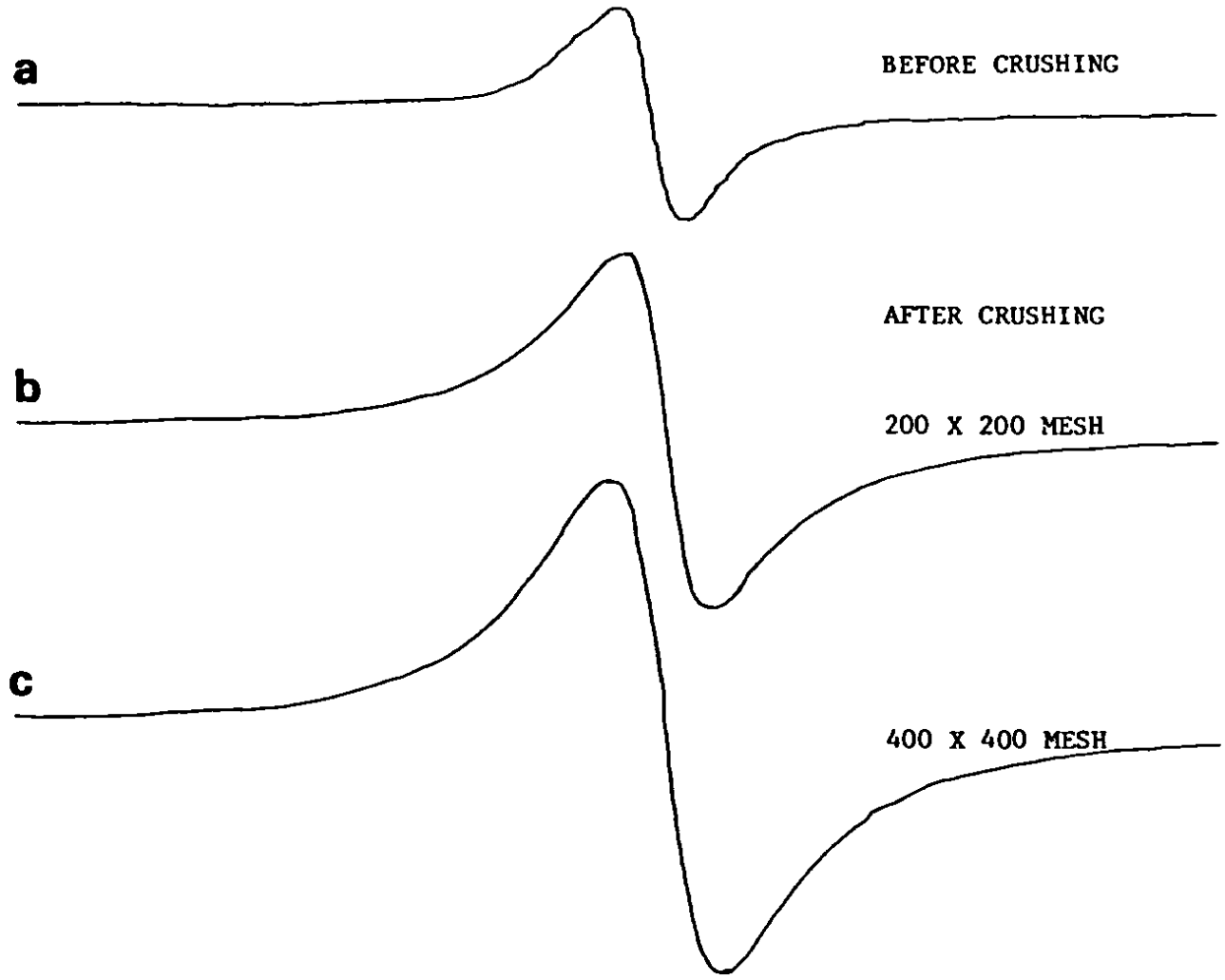

Figure 1. Time dependence of the decay of radicals in air.

Table I

Effect of Crushing on the Anthracite (PSOC-867) Coal		
	Size	Spin/Gram
Before Crushing		4.8×10^{16}
After Crushing	200 x 200 mesh	1.5×10^{17}
Before Crushing		7.9×10^{16}
After Crushing	400 x 400 mesh	4.3×10^{17}

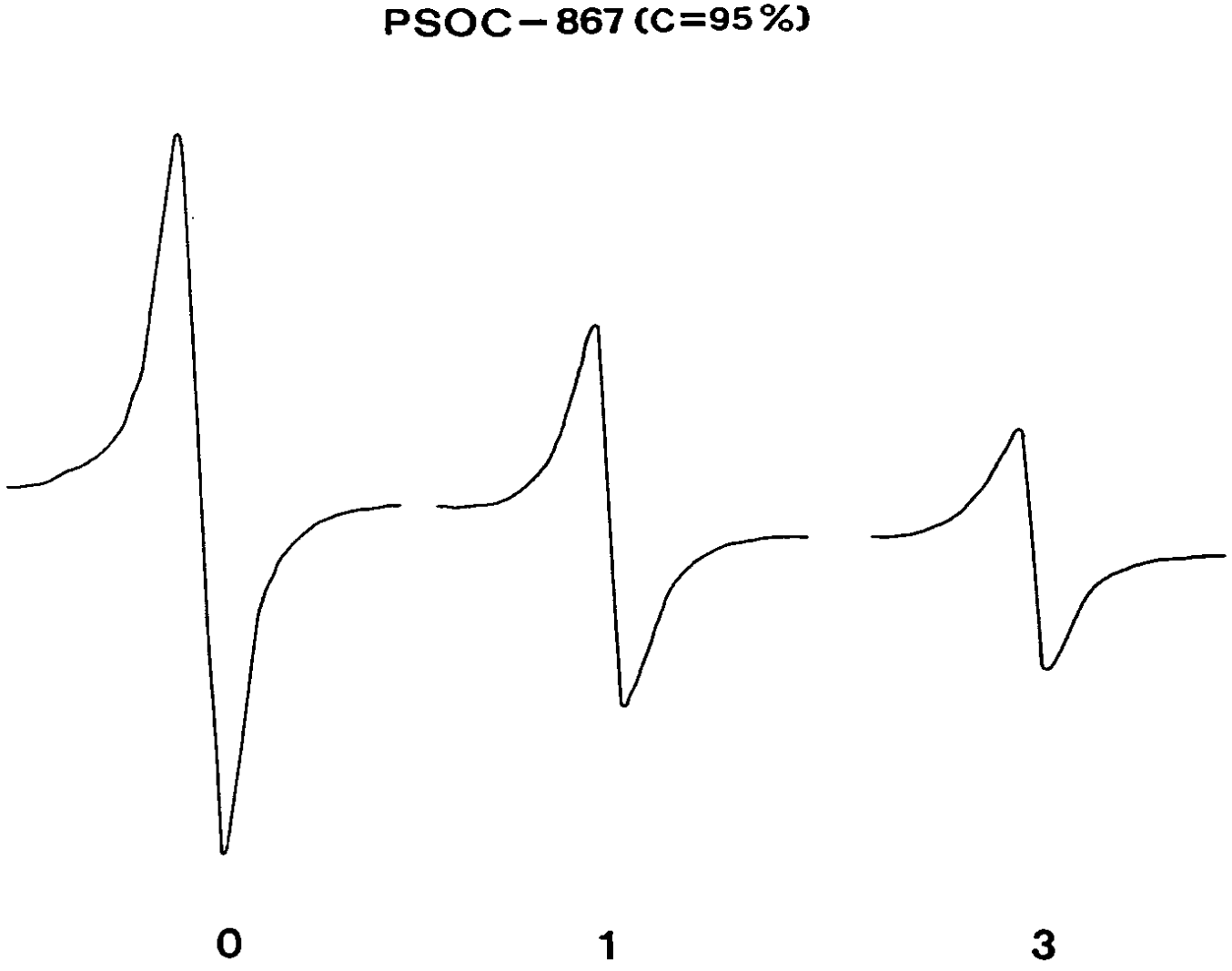

Figure 2. ESR spectra showing the formation of radicals on crushing.

Sodium Benzoate

As a third test, we investigated the hemolytic activity of the fresh dust in the presence of sodium benzoate, a compound often used tp specifically quench ·OH radicals in biological systems.[14] Sodium benzoate was added at two different concentrations, 0.1 mg/4 ml and 0.01 mg/4 ml. The results are presented in Table IV wherefrom it is clear that sodium benzoate decreases the hemolytic activity in a dose-response manner but with much less efficiency than catalase or SOD. These results suggest that the ·OH radicals are not the main species in the mechanism of the membrane cytotoxicity of the fresh anthracite dusts.

Table II

Decay of Radicals in an Anthracite Coal Crushed and Kept in Air	
Time (hours)	Spins/Gram
0	4.28×10^{17}
10	4.33×10^{17}
20	4.44×10^{17}
50	4.31×10^{17}
100	4.07×10^{17}
170	3.35×10^{17}

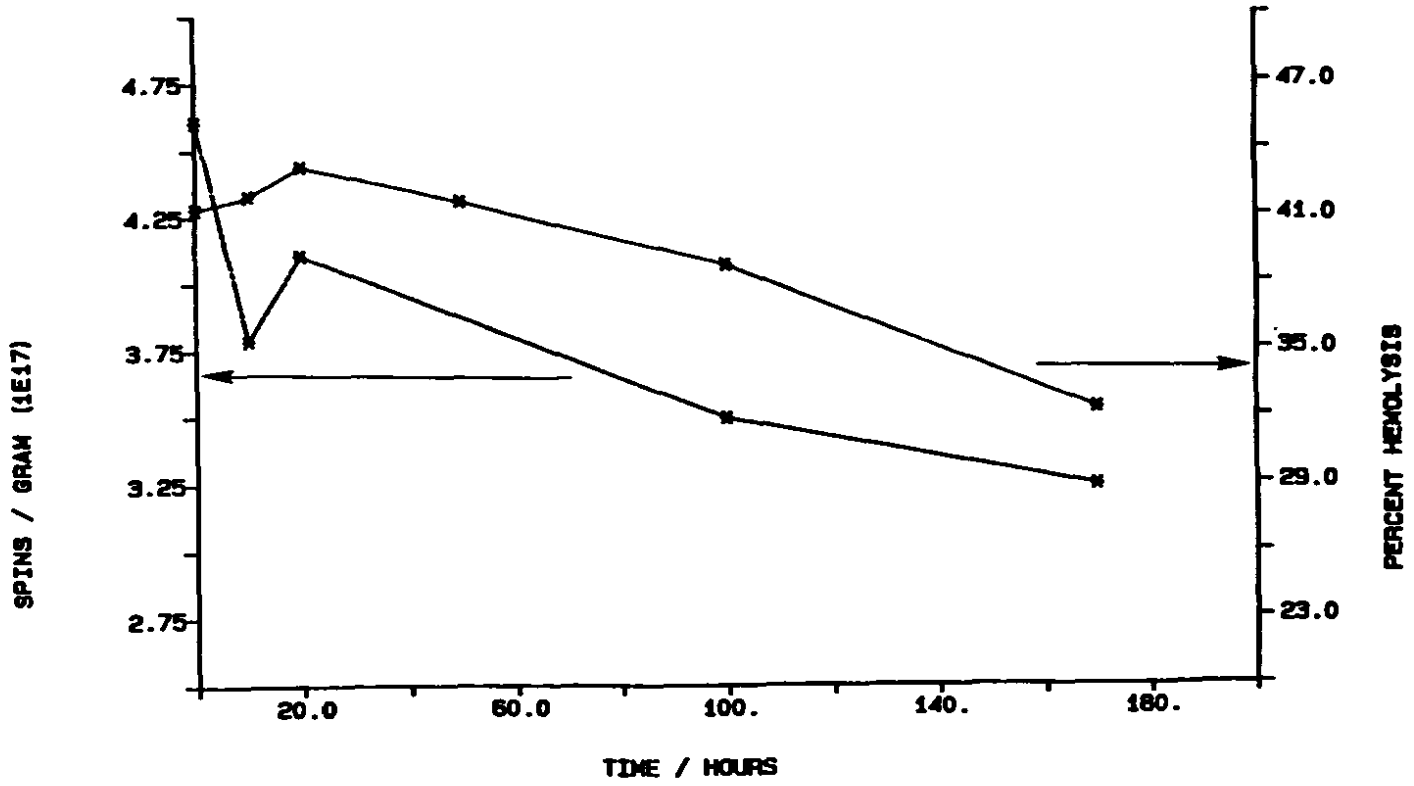

Figure 3. Correlation of the radical concentration measured by ESR, and the toxicity deduced from hemolysis studies of fresh anthracite coal as a function of time.

Table III

Percent Hemolysis Data for an Anthracite Coal (PSOC-867) Crushed in Air

Time/Hours	Percent Hemolysis			
	5 mg/ml AIR	5 mg/ml BUFFER	10 mg/ml AIR	10 mg/ml BUFFER
0	24.5	24.5	45.3	45.3
4	18.5	20.0	35.5	37.5
24	20	21.7	39.3	41.0
96	17.3	20.0	32.0	40.0
176	17.0	20.5	29.0	40.0

Table IV

Effect of SOD, Catalase, and Sodium Benzoate on Hemolysis of Coal Dust

Compound	Concentration	Percent Hemolysis
SOD	5 mg/ml	31.1
	5 mg/ml + SOD	8.7
	10 mg/ml	46.3
	10 mg/ml + SOD	24.3
Catalase	5 mg/ml	29.7
	5 mg/ml + Catalase	10.6
	10 mg/ml	47.2
	10 mg/ml + Catalase	23.3
Sodium Benzoate	5 mg/ml	32.5
	5 mg/ml + 0.1 mg Sodium Benzoate	22.9
	5 mg/ml + 0.1 mg	26.5
	10 mg/ml	45.3
	10 mg/ml + 0.1 mg Sodium Benzoate	31.9
	10 mg/ml + 0.01 mg Sodium Benzoate	38.4

Oxygen Atmosphere

In order to further ascertain if oxygen plays a direct role in the cytotoxicity, not involving the mechanism of any oxygenated species, we carried out comparative hemolysis studies of coal dusts particles under flowing nitrogen gas (to exclude oxygen) and, separately, in air. Moreover the measurements were made for two different particle sizes, 200 x 200 mesh (<40 microns) and 400 by 400 (<25 microns). As shown in Figure 4. The results show that the participation of oxygen is as important to the mechanism of the fresh dust's cytotoxicity as measured by hemolysis, in conformity with the conclusions from the above discussed measurements employing the oxygen radical quenchers.

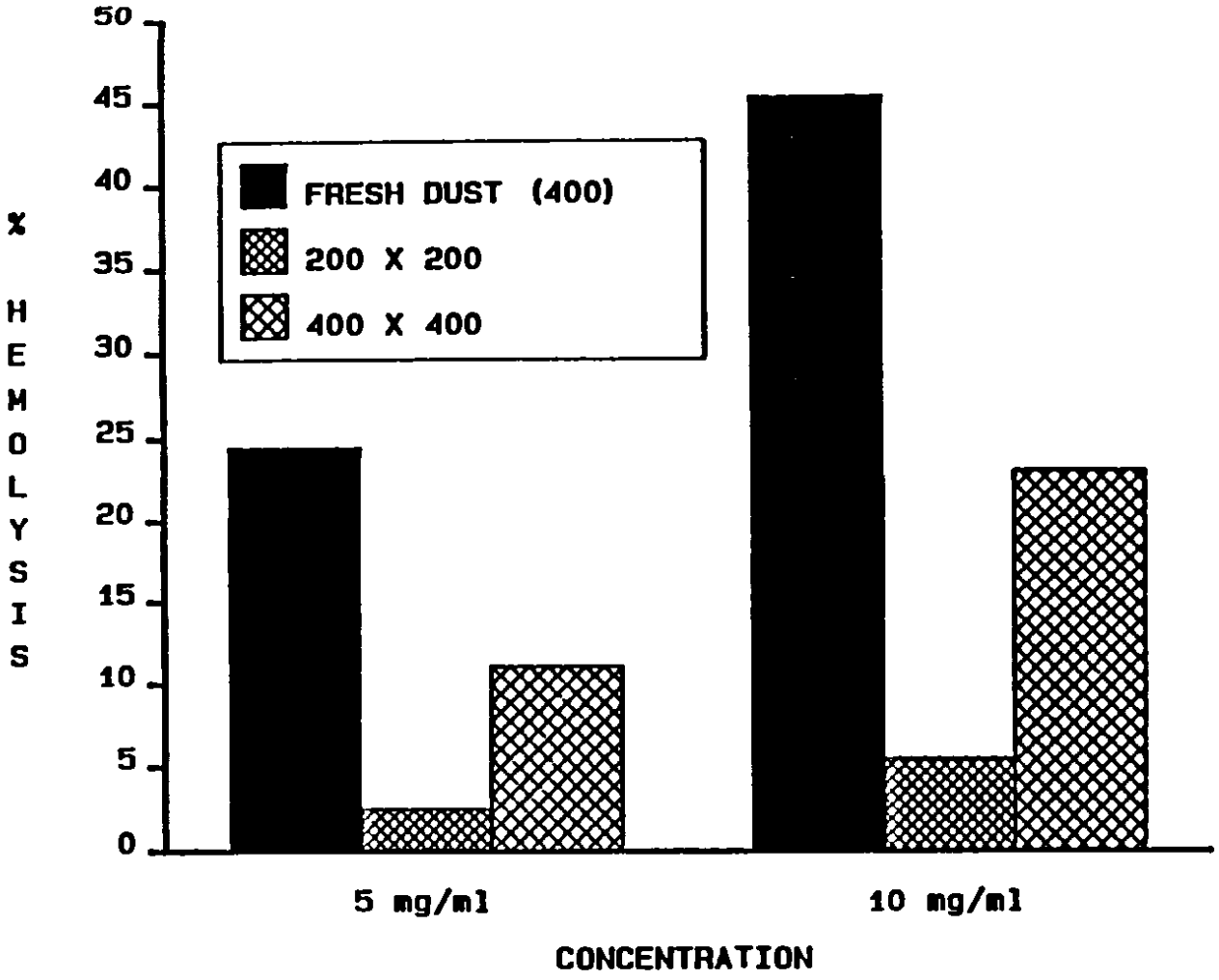

Figure 4. Effect of particle size and nitrogen atmosphere on the hemolysis by the fresh anthracite dust. The data indicates mean of 7 experiments with freshly prepared dust.

Conclusions

The above results suggest that freshly made anthracite coal dusts are more cytotoxic than the 'stale' dusts from the same stock, and that surface oxidation reactions involving free radical sites on the coal particles play a significant role in the dusts cytotoxicity.

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